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COMISIÓN DE NORMAS BIOLÓGICAS

Propuesta de enmiendas al
*Manual de las Pruebas de Diagnóstico y de las Vacunas para los
Animales Terrestres*

Documento de trabajo técnico



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I. Introducción

Desde la 90ª Sesión General, celebrada en mayo de 2023, la Comisión de Normas Biológicas se ha reunido dos veces, del 4 al 8 de septiembre de 2023 y del 5 al 9 de febrero de 2024. Entre sus actividades, la Comisión ha avanzado en la elaboración de textos nuevos y revisados para el *Manual de las Pruebas de Diagnóstico y de las Vacunas para los Animales Terrestres (Manual Terrestre)*, de acuerdo con su programa de trabajo. Los pormenores de las actividades de la Comisión, incluidos los textos distribuidos para recabar comentarios, se publicaron en los informes de las reuniones de septiembre de 2023 y febrero de 2024 de la Comisión y se pueden consultar en el sitio web de los Delegados, así como en la [página de la OMSA](#).

Este informe ofrece un breve resumen de cada uno de los textos revisados del *Manual Terrestre* que se presentarán para adopción en la 91ª Sesión General. En los informes de las reuniones de la Comisión de [septiembre de 2023](#) y de [febrero de 2024](#) se detallan los comentarios de los Miembros sobre los borradores. La Comisión anima a los Miembros a remitirse a estos informes de reunión para obtener más detalles sobre los textos enmendados que se propondrán para adopción.

Los anexos a este documento presentan las enmiendas propuestas a los capítulos del *Manual Terrestre* que se someterán a la aprobación de la Asamblea Mundial de Delegados en la 91ª Sesión General. Los números de los anexos coinciden con los que figuran en el informe de febrero de 2024 de la Comisión de Normas Biológicas.

Es posible que durante la Sesión General se propongan otras enmiendas al borrador basadas en los comentarios de los Miembros recibidos durante la segunda ronda de comentarios (fecha límite: [30 de abril de 2024](#)).

En el proceso de redacción y revisión de estas enmiendas, la Comisión tuvo en cuenta los comentarios enviados por los Miembros y por las Organizaciones Internacionales que tienen un acuerdo de cooperación con la OMSA.

1. Textos del *Manual Terrestre* propuestos para adopción

1.1 *Capítulo 1.1.5. «Gestión de la calidad en los laboratorios de pruebas veterinarias»* ([Anexo 4](#))

El Capítulo 1.1.5. «Gestión de la calidad en los laboratorios de pruebas veterinarias» ha sido objeto de una revisión exhaustiva. El texto revisado se distribuyó para una primera ronda de comentarios en octubre de 2023. Las principales modificaciones incluyen: actualización de las referencias y URL; aclaración de que una escasa disponibilidad de material adecuado puede dificultar la validación, y el traslado a la sección A.7.3 «Validación del método analítico»; y actualizaciones técnicas sustanciales en los apartados sobre:

- Acreditación;
- Determinación del alcance del sistema de gestión de la calidad o de la acreditación del laboratorio;
- Validación del método analítico;
- Estimación de la incertidumbre de la medición.

Y actualización del apartado sobre planificación estratégica.

1.2 *Capítulo 1.1.9. «Pruebas de esterilidad y ausencia de contaminación en los materiales biológicos de uso veterinario»* ([Anexo 5](#))

El Capítulo 1.1.9. «Pruebas de esterilidad y ausencia de contaminación de los materiales biológicos de uso veterinario» ha sido objeto de una revisión exhaustiva. El texto revisado se distribuyó para una primera ronda de comentarios en octubre de 2023. Las principales modificaciones son las siguientes: actualización para ofrecer una visión general basada en ejemplos de las pruebas y sus antecedentes normativos, incluidos breves ejemplos ilustrativos de contaminación de vacunas; adición de datos a la sección G. «Ejemplos de protocolos» y una clara adjetivación de los ejemplos como no prescriptivos y no exhaustivos, ya que son un fuerte incentivo para las pruebas de agentes extraños; actualización de la sección A. «Revisión de los métodos analíticos» destacando las oportunidades y desafíos más recientes; fusión de las secciones sobre virus vivos e inactivados y bacterias, para simplificar y racionalizar el capítulo; y actualización de la bibliografía y los enlaces web.

1.3 *Capítulo 2.2.4. «Incertidumbre de la medición»* ([Anexo 6](#))

El Capítulo 2.2.4. «Incertidumbre de la medición» ha sido objeto de una revisión exhaustiva. El texto revisado se distribuyó para una primera ronda de comentarios en octubre de 2023. Las principales modificaciones son las siguientes: supresión de la referencia a la «Norma de validación de la OMSA»: el capítulo 1.1.6 diferirá del futuro capítulo del *Manual Acuático*, por lo que ya no existe una norma que se aplique a ambos Manuales; aclaración de que el método descrito en este capítulo se conoce como método «descendente», e inclusión de información sobre sus requisitos junto con una sección sobre el alcance y las limitaciones del método descendente; aclaración de que existen métodos alternativos que dependen menos de hipótesis de distribución y que permiten gestionar mejor la presencia de valores atípicos; adición de un ejemplo de cálculo de la incertidumbre de la medición en pruebas moleculares.

1.4 Capítulo 2.2.6. «Elección y uso de tipos y grupos de muestras de referencia» ([Anexo 7](#))

El capítulo 2.2.6. «Elección y uso de muestras y grupos de muestras de referencia» ha sido objeto de una revisión moderada. El texto revisado se distribuyó para una primera ronda de comentarios en octubre de 2023. Las principales modificaciones son: actualización de las referencias cruzadas con el Capítulo 1.1.6 «Validación de las pruebas de diagnóstico de las enfermedades infecciosas de animales terrestres»; adición de una figura sobre la documentación que se exige a los materiales de referencia; adición de una lista de referencias para lecturas complementarias; adición de una sección sobre «Lecturas complementarias» con bibliografía revisada por expertos.

1.5 Capítulo 3.1.5. «Fiebre hemorrágica de Crimea–Congo» ([Anexo 8](#))

El Capítulo 3.1.5. «Fiebre hemorrágica de Crimea–Congo» ha sido objeto de una revisión moderada. El texto revisado se distribuyó para una primera ronda de comentarios en octubre de 2023. Las principales modificaciones son: adición de dos notas a pie de página a la finalidad «Confirmación de casos clínicos en animales» en la Tabla 1 «Formatos de pruebas de diagnóstico para las infecciones por el virus de la fiebre hemorrágica de Crimea–Congo en animales» para garantizar que concuerda con la definición de caso: las notas a pie de página serán reemplazadas por un enlace a la definición de caso una vez que el capítulo sea adoptado e incluido en el *Código Terrestre*; cambio en la clasificación de la PCR en tiempo real para la finalidad «Demostrar ausencia de infección en animales individuales antes de los desplazamientos», ya que la investigación sobre el VFHCC confirma la viremia transitoria.

1.6 Capítulo 3.3.6. «Tuberculosis aviar» ([Anexo 9](#))

El Capítulo 3.3.6. «Tuberculosis aviar» ha sido objeto de una revisión exhaustiva. El texto revisado se distribuyó para una primera ronda de comentarios en octubre de 2023. Las principales modificaciones son las siguientes: actualización de la nomenclatura y la clasificación de las especies del género *Mycobacterium*; revisión de las calificaciones de algunas de las pruebas de la Tabla 1 «Métodos analíticos disponibles para el diagnóstico de la tuberculosis aviar y su propósito»; actualización de la sección sobre los métodos de reconocimiento de ácidos nucleicos; adición de una sección sobre la prueba del antígeno teñido; actualización de la sección sobre la producción y los requisitos mínimos para la producción de tuberculina; actualización de la bibliografía. **NB:** Dado que la tuberculosis aviar no figura en la lista de enfermedades de la OMSA, este capítulo se suprimirá del *Manual Terrestre*; una vez adoptado, la información sobre la producción de tuberculina aviar se trasladará al Capítulo 3.1.13 «Tuberculosis de los mamíferos (infección por el complejo *Mycobacterium tuberculosis*)».

1.7 Capítulo 3.4.1. «Anaplasmosis bovina» ([Anexo 10](#))

El Capítulo 3.4.1. «Anaplasmosis bovina» ha sido objeto de una revisión exhaustiva. El texto revisado se distribuyó para una primera ronda de comentarios en octubre de 2023. Las principales modificaciones incluyen: actualización de la información en la introducción del capítulo; adición de una ilustración de un frotis sanguíneo teñido que muestra cuerpos de inclusión de *Anaplasma marginale*; actualización exhaustiva de la sección sobre la PCR, incluida la adición de una tabla de secuencias de cebadores, y de la sección sobre los ELISA, incluida la adición de un ELISA sándwich de doble antígeno de desplazamiento que se ha desarrollado para diferenciar entre los anticuerpos contra *A. marginale* y contra *A. centrale*; revisión de las clasificaciones de algunas de las pruebas de la Tabla 1 «Métodos analíticos disponibles para el diagnóstico de la anaplasmosis bovina y su propósito»; se ha subrayado que la prueba de fijación del complemento tiene una sensibilidad variable y se ha eliminado de la Tabla 1. Este capítulo incluye ahora tablas de justificación de las calificaciones de las pruebas de la Tabla 1 para cada finalidad: estas tablas de justificación serán de gran utilidad para los usuarios del *Manual Terrestre* a la hora de decidir qué prueba utilizar para una finalidad determinada.

1.8 Capítulo 3.4.7. «Diarrea viral bovina» ([Anexo 11](#))

El Capítulo 3.4.7. «Diarrea viral bovina» ha sido objeto de una revisión moderada. El texto revisado se distribuyó para una primera ronda de comentarios en octubre de 2023. Las principales modificaciones incluyen: actualización de la taxonomía; revisión de las calificaciones de algunas de las pruebas de la Tabla 1 «Métodos analíticos disponibles para el diagnóstico de la diarrea viral bovina y su propósito»; elaboración de tablas de justificación de las calificaciones de las pruebas de la Tabla 1 para cada finalidad.

1.9 Capítulo 3.4.12. «Dermatosis nodular contagiosa» (solo la sección sobre vacunas) ([Anexo 12](#))

El Capítulo 3.4.12. «Dermatosis nodular contagiosa» (sólo la sección sobre vacunas) ha sido objeto de una revisión exhaustiva. El texto revisado se distribuyó para una primera ronda de comentarios en octubre de 2023. Las principales modificaciones incluyen: adición de texto sobre la escasez de información acerca del papel de la fauna silvestre en la epidemiología de la dermatosis nodular contagiosa; y una actualización completa de la sección C «Requisitos para las vacunas».

1.10 Capítulo 3.6.9. «Rinoneumonía equina (infección por Varicellovirus equidalpha1)» [formalmente, herpesvirus-1 de los équidos] ([Anexo 13](#))

El Capítulo 3.6.9. «Rinoneumonitis equina (infección por Varicellovirus equidalpha1)» [formalmente, herpesvirus-1 de los équidos] ha sido objeto de una revisión exhaustiva. El texto revisado se distribuyó para una primera ronda de comentarios en octubre de 2023. Las principales modificaciones consisten en: una actualización de la taxonomía del agente patógeno, puesto que el herpesvirus-1 de los équidos ahora se denomina Varicellovirus equidalpha1, y como el capítulo trata de la infección por Varicellovirus equidalpha1, se ha suprimido la mayor parte de la información sobre el herpesvirus-4 de los équidos, ya que el HVE4 no figura en la lista; una actualización exhaustiva de la Sección B «Técnicas de diagnóstico», en particular el apartado sobre detección del virus mediante PCR, que ahora cuenta con una tabla sobre secuencias de cebadores y sondas para una serie de PCR en tiempo real, subsecciones sobre pruebas moleculares POC y caracterización molecular, secciones sobre aislamiento de virus y neutralización de virus, y una sección sobre la prueba de fijación del complemento; elaboración de tablas de justificación para las calificaciones de las pruebas de la Tabla 1 para cada finalidad.

1.11 Capítulo 3.8.1. «Enfermedad de la frontera» ([Anexo 14](#))

El Capítulo 3.8.1. «Enfermedad de la frontera» ha sido objeto de muy poca revisión. El texto revisado se distribuyó para una primera ronda de comentarios en octubre de 2023. Las principales modificaciones son: una actualización menor, principalmente de la taxonomía.

1.12 Capítulo 3.8.12. «Viruela ovina y viruela caprina» (solo la sección sobre pruebas de diagnóstico) ([Anexo 15](#))

El Capítulo 3.8.12. «Viruela ovina y viruela caprina» (sólo la sección sobre pruebas de diagnóstico) ha sido objeto de una revisión moderada. El texto revisado se distribuyó para una primera ronda de comentarios en octubre de 2023. Las principales modificaciones son las siguientes: inclusión de las pruebas de anticuerpos fluorescentes, la histopatología y el ELISA en la Tabla 1 «Métodos analíticos disponibles para el diagnóstico de la viruela ovina y la caprina y su finalidad»; actualización completa de la sección sobre métodos de reconocimiento de ácidos nucleicos, en particular los métodos de PCR convencional y en tiempo real; aclaración de que las pruebas ELISA no pueden discriminar entre anticuerpos contra diferentes capripoxvirus.

1.13 Capítulo 3.9.1. Peste porcina africana (solo la sección sobre vacunas) ([Anexo 16](#))

El Capítulo 3.9.1. «Peste porcina africana» (solo la sección sobre vacunas) ha sido objeto de una revisión exhaustiva. El texto revisado se distribuyó para una primera ronda de comentarios en octubre de 2023. Las principales modificaciones incluyen: actualización de la sección C «Requisitos para las vacunas» sobre la fabricación de vacunas puras, potentes, inocuas y eficaces contra la PPA, incluidos los criterios clave de rendimiento y calidad de las vacunas, tras consultar con fabricantes de vacunas, expertos en la materia, representantes de la comunidad científica, autoridades reguladoras y Laboratorios de Referencia de la OMSA. En el informe de septiembre de 2023 también se incluyó, a título informativo, un apéndice del borrador de capítulo con los resultados de la consulta, los parámetros clave, resúmenes de los debates, justificaciones científicas, etc.

Annexe 4. Chapter 1.1.5. 'Quality management in veterinary testing laboratories'

CHAPTER 1.1.5.

QUALITY MANAGEMENT IN VETERINARY TESTING LABORATORIES

SUMMARY

Valid laboratory results are essential for diagnosis, surveillance, and trade. Such results are achieved by the use of assured through implementation of good a management practices, valid system that supports accurate and consistent test and calibration methods, proper techniques, quality control and quality assurance, all working together within a quality management system. Laboratory quality management includes technical, managerial, and operational elements of testing-performing, interpreting and the interpretation of reporting test results. A quality management system enables the laboratory to demonstrate both competency and an ability to generate consistent technically valid results that meet the needs of its customers. The need for Mutual recognition and acceptance of test results for international trade, and the acceptance accreditation of tests to international standards such as ISO/IEC¹ 17025-2005 (General Requirements for the Competence of Testing and Calibration Laboratories) (ISO/IEC, 2005-2017b) requires good-suitable laboratory quality management systems. This chapter is not intended to reiterate the requirements of ISO/IEC 17025, nor has it been endorsed by accreditation bodies. Rather, it outlines the important issues and considerations a laboratory should address in the design and maintenance of its quality management system, whether or not it has been formally accredited regardless of formal accreditation status. Chapter 1.1.1 Management of veterinary diagnostic laboratories gives an introduction to veterinary diagnostic laboratories introduces the components of governance and management of veterinary laboratories that are necessary for the effective delivery of diagnostic services, and highlights the critical elements that should be established as minimum requirements.

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

To ensure that the quality management system is appropriate and effective, the design must be carefully thought out-planned and, where accreditation is sought, must address all criteria of the appropriate quality standard. The major categories of considerations and the their associated key issues and activities within each of these categories are outlined in the following eight sections of this chapter.

¹ ISO/IEC: International Organization for Standardization/International Electrochemical Commission.

1. The work, responsibilities, and goals of the laboratory

Many factors affect the necessary elements and requirements of a quality management system. ~~These factors include, including:~~

- i) ~~Type of testing done performed,~~ e.g. research versus diagnostic work;
- ii) Purpose and requirements of ~~the test results,~~ e.g. ~~for import or export quarantine testing, surveillance, emergency disease exclusion, declaration of freedom from disease post-outbreak;~~
- iii) Potential impact of a questionable ~~or,~~ erroneous or unfavourable result, e.g. detection of foot and mouth disease (FMD) in an FMD-free country;
- iv) ~~The tolerance level of Risk and liability~~ tolerance, e.g. vaccination ~~vs versus~~ culling ~~or slaughter;~~
- v) Customer ~~needs (requirements,~~ e.g. sensitivity and specificity ~~of the test method,~~ cost, turnaround time, ~~strain or genotype level of~~ characterisation), e.g. ~~for surveillance, or declaration of freedom after outbreak;~~
- vi) ~~The role of the laboratory~~ Role in legal work or in regulatory programmes, e.g. for disease eradication and declaration of disease freedom to the WOAHP;
- vii) ~~The role of the laboratory~~ Role in assisting with, confirming, or overseeing the work of other laboratories (e.g. as a reference laboratory);
- viii) Business goals ~~of the laboratory,~~ including the need for any third-party recognition or accreditation.

2. Standards, guides, and references

The laboratory should ~~choose reputable and accepted~~ follow globally recognised standards and guides to assist in designing the quality management system. For laboratories seeking ~~accreditation formal recognition of testing competency,~~ and for all WOAHP Reference Laboratories, the use of ISO/IEC 17025 (ISO/IEC, 2005-2017b) or equivalent ~~will be is~~ essential. This standard ~~includes specifies~~ managerial and technical requirements and accredited laboratories ~~that are compliant~~ are regarded as competent. Further information on standards may be obtained from the national standards body of each country, from the International Laboratory Accreditation Cooperation (ILAC)², and from accreditation bodies, e.g. ~~the National Association of Testing Authorities (NATA), Australia, the United Kingdom Accreditation Service (UKAS), the American Association for Laboratory Accreditation (A2LA), etc.~~ Technical and international organisations such as AOAC International (The Scientific Association Dedicated to Analytical Excellence; formerly the Association of Official Analytical Chemists) and the International Organization for Standardization (ISO) publish useful references, guides, application documents and standards that supplement the general requirements of ISO/IEC 17025. Other relevant documents may include guide <https://nata.com.au/files/2021/05/Animal-Health-ISO-IEC-17025-Appendix-effective-March2021.pdf>; Newberry & Colling, 2021.

The ISO International Standard 9001 (ISO, 2015), ~~is a certification standard~~ specifies the requirements for quality management systems and while it may be a useful ~~supplement framework to a~~ underpin a laboratory quality system, fulfilment of its requirements ~~does not necessarily ensure or imply assure~~ technical competence (in the areas listed in Section 3 *Accreditation*). Conformance to the requirements of ISO 9001 is assessed by a certification body that is accredited ~~to undertake such assessments by~~ the national accreditation body to undertake such assessments. When a laboratory meets the requirements of ISO 9001, the term *registration* or *certification* is used to indicate conformity, not *accreditation*.

With the advent of stronger alliances between medical and veterinary diagnostic testing under initiatives such as “One Health”, some laboratories may ~~wish to choose to follow~~ other ISO standards such as ISO 15189 Medical Laboratories – Requirements for Quality and Competence (ISO/IEC, 2012), which include 2022), for testing of human samples, e.g. for zoonotic diseases. It should be noted that for veterinary laboratories, limited availability of suitable material may render validation difficult, under these

² ILAC: The ILAC Secretariat, PO Box 7507, Silverwater, NSW 2128, Australia; <http://ilac.org/>

78 circumstances it is necessary to highlight the limited validation status when reporting results and their
79 interpretation (Stevenson *et al.*, 2021).

80 3. Accreditation

81 If ~~the laboratory decides to proceed with~~ formal recognition of ~~its a laboratory's~~ quality management
82 system and testing, ~~then is sought~~, third party verification of its conformity with the selected standard(s)
83 ~~will be is~~ necessary. ILAC has published specific requirements and guides for laboratories and
84 accreditation bodies. Under the ILAC system, ISO/IEC 17025 is to be used for laboratory accreditation
85 of testing or calibration activities. Definitions regarding laboratory accreditation may be found in ISO/IEC
86 International Standard 17000: Conformity Assessment – Vocabulary and General Principles (ISO/IEC,
87 2004a-2020). Accreditation is not tied to dependent on demonstrated competence, which is encompasses
88 significantly more than having and following documented procedures. Providing a competent and
89 customer-oriented service also ~~means that the laboratory requires~~:

- 90 i) Adequate facilities and environmental controls;
- 91 ii) ~~Has~~ Appropriately qualified and trained personnel with a depth of technical knowledge
92 commensurate with appropriate level of authority;
- 93 iii) ~~Has appropriate~~ Equipment with ~~planned that is~~ appropriately verified and managed in accordance
94 with the relevant maintenance and calibration schedule;
- 95 iv) ~~Has adequate facilities and environmental control;~~
- 96 v) ~~Has procedures and specifications that ensure accurate and reliable results;~~
- 97 vi) ~~Implements continual improvements in testing and quality management;~~
- 98 vii) ~~Can assess the need for and implement appropriate corrective or preventive actions, e.g. customer~~
99 ~~satisfaction;~~
- 100 viii) ~~Accurately assesses and controls uncertainty in testing;~~
- 101 iv) Appropriate sample and materials management processes;
- 102 v) ~~Has~~ Technically valid and validated test methods, procedures and specifications ~~that are,~~
103 documented in accordance with the requirements of the applicable standard or guidelines, e.g.
104 Chapter 1.1.6 *Principles and methods of validation of diagnostic assays for infectious diseases*
105 ~~and~~, chapters 2.2.1 to 2.2.8 *Recommendations for validation of diagnostic tests* and Special Issue
106 of the Scientific and Technical Review (2021)³;
- 107 vi) ~~Demonstrates~~ Demonstrable proficiency in the applicable test methods used ~~(e.g. by regular~~
108 ~~participation in proficiency tests on a regular basis testing schemes);~~
- 109 vii) Accurate assessment and control of the measurement of uncertainty in testing;
- 110 viii) Good documentation practices, e.g. ALCOA+ principles (i.e. Attributable, Legible,
111 Contemporaneous, Original, Accurate, Complete, Consistent, Enduring, Available);
- 112 ix) Non-conformance management process, including detection, reporting, risk-assessment and
113 implementation of effective corrective and preventive actions;
- 114 x) Complaints management;
- 115 xi) Adequate control of data and information;
- 116 xii) Appropriate reporting and approval process;
- 117 xiii) Culture of continual improvement.
- 118 xiv) Has demonstrable competence to generate technically valid results.

³ Available at: <https://doc.woaah.org/dyn/portal/index.xhtml?page=alo&alold=41245>

119 **4. Selection of an accreditation body**

120 To facilitate the acceptance of the laboratory's test results for trade, the accreditation standard used
121 must be recognised by the international community and the accreditation body recognised as competent
122 to accredit laboratories. Programmes for the recognition of accreditation bodies are, in the ILAC scheme,
123 based on the requirements of ISO/IEC International Standard 17011: Conformity Assessment – General
124 Requirements for Accreditation Bodies Accrediting Conformity Assessment Bodies (ISO/IEC, 2004b
125 2017a). Information on recognised accreditation bodies may be obtained from the organisations that
126 recognise them, such as the Asia-Pacific Accreditation Cooperation (APAC), the Inter-American
127 Accreditation Cooperation (IAAC), and the European Co-operation for Accreditation (EA).

128 Accreditation bodies may also be signatory to the ILAC and regional (e.g. APAC) mutual recognition
129 arrangements (MRAs). These MRAs are designed to reduce technical barriers to trade and further
130 facilitate the acceptance of a laboratory's test results in foreign markets. Further information on the ILAC
131 MRA may be obtained from the <http://www.ilac.org>.

132 **5. Determination of the scope of the quality management system or of the laboratory's**
133 **accreditation**

134 ~~The scope of the~~ quality management system should ~~cover all areas of activity affecting all~~ include all
135 activities that impact testing ~~that is done at~~ performed by the laboratory. Whilst only accredited
136 laboratories are obliged to meet the requirements of the relevant standard ~~as detailed below, these, the~~
137 guiding principles should be considered best practise and are relevant to all testing laboratories.

138 ~~Laboratories accredited~~ A laboratory's accreditation to ISO/IEC 17025 ~~have~~ includes a specific list of
139 ~~these accredited~~ tests ~~that are accredited, called, referred to as~~ the schedule or scope of accreditation
140 ~~or the scope~~. Veterinary testing facilities include government and private facilities, veterinary practices,
141 university veterinary schools, and other laboratories for the testing of animals and animal products for
142 the diagnosis, monitoring and treatment of disease. In principle, if new testing methods are introduced
143 these must be assessed and accredited before they can be added to the scope, however a flexible
144 scope can be implemented that assesses the laboratory as competent to add tests to scope, which are
145 then formally added at the next accreditation visit. ~~The quality management system should ideally cover~~
146 ~~all areas of activity affecting all testing that is done at~~ the laboratory. However, it is up to the laboratory
147 ~~to decide which tests are to be accredited and included in the scope~~. If an accredited laboratory also
148 offers ~~unaccredited~~ non-accredited tests, these must be clearly indicated as such on any reports that
149 claim ~~or make~~ reference to accreditation. ~~Factors~~ It is ultimately the decision of the laboratory to decide
150 which tests require inclusion in the scope of accreditation, and factors that might affect ~~the laboratory's~~
151 ~~choice of tests for scope of accreditation~~ this decision include:

- 152 i) ~~The impact of initial accreditation on resources within a given deadline;~~
153 i) Associated risks and opportunities;
154 ii) Initial investment required (e.g. time, resources);
155 ~~iii) A~~ Contractual requirement for accredited testing (e.g. for international trade, research projects);
156 ~~iv) The~~ Importance of the test and the potential impact of an incorrect result;
157 v) The cost of maintaining an accredited test versus frequency of use;
158 vi) Availability of personnel, facilities and equipment;
159 vii) Availability of appropriate materials and reference standards (e.g. ~~standardised~~ reagents, ~~internal~~
160 ~~quality control samples~~ controls, reference cultures) ~~and~~
161 viii) Access to proficiency testing schemes;
162 ix) The quality ~~assurance~~ control processes necessary for materials, reagents and media;
163 x) The validation status, e.g. access to field samples from infected and non-infected animals, technical
164 complexity and reliability of the test method;
165 xi) ~~The~~ Potential for subcontracting of accredited tests.

166 **6. Quality assurance, quality control and proficiency testing**

167 Quality assurance (QA) is the ~~part element~~ of quality management focused on providing confidence that
168 ~~quality defined~~ requirements ~~will be~~ are fulfilled. The requirements may be internal or defined in an
169 accreditation or certification standard. QA is process-oriented and ~~ensures provides~~ the right things are
170 ~~being done in the right way~~ appropriate inputs to prevent problems arising.

171 Quality control (QC) is the systematic and planned monitoring of outputs to ensure ~~the minimum levels~~
172 ~~of quality requirements~~ have been met. For a testing laboratory, this is ~~to ensure test processes ensures~~
173 ~~tests are working correctly performing consistently and reliably~~, and results are within ~~the expected~~
174 ~~acceptable~~ parameters and limits. QC is ~~test orientated and ensures the results are as expected~~
175 ~~oriented and ensures detection of any problems that arise~~.

176 Proficiency testing (PT), sometimes referred to as external quality assurance ~~or~~ (EQA), is the
177 ~~determination assessment~~ of a laboratory's performance ~~by when~~ testing a standardised panel of
178 specimens of undisclosed content. Ideally, PT schemes should be ~~run managed~~ by an external
179 independent provider. Participation in proficiency testing schemes enables the laboratory to assess and
180 demonstrate ~~the their testing~~ reliability of results ~~by in~~ comparison with ~~those from~~ other participating
181 laboratories.

182 All laboratories should, where possible, participate in external proficiency testing schemes appropriate
183 ~~to their testing. Participation the suite of tests provided; participation~~ in such schemes is a requirement
184 for accredited laboratories. This provides an independent assessment of the testing methods used ~~and~~
185 as well as the level of staff competence. If such schemes are not available, valid alternatives may be
186 used, such as ring trials organised by reference laboratories, inter-laboratory testing, use of certified
187 reference materials or internal quality control samples, replicate testing using the same or different
188 methods, retesting of retained items, ~~and or~~ correlation of results for different characteristics of a
189 specimen.

190 Providers and operators of proficiency testing programmes should be accredited to ISO/IEC 17043 –
191 Conformity Assessment – General Requirements for Proficiency Testing (ISO/IEC, 2010).

192 Proficiency testing material from accredited providers ~~has been~~ is well characterised and any spare
193 material, once the proficiency testing has been completed, can be useful to demonstrate staff
194 competence or for test validation. Information about selection and use of reference samples and panels
195 is available in Chapter 2.2.6 *Selection and use of reference samples and panels*. Proficiency testing and
196 reproducibility scenarios are described by Johnson & Cabuang (2021) and Waugh & Clark (2021),
197 respectively.

198 **7. Test methods**

199 ISO/IEC 17025 requires the use of appropriate test methods and has requirements for their selection,
200 development, and validation to ~~show demonstrate~~ fitness for purpose.

201 This *Terrestrial Manual* provides recommendations on the selection of test methods for trade, diagnostic
202 and surveillance purposes in the chapters on specific diseases. Disease-specific chapters include, or
203 will include in the near future, a table of the tests available for the disease, graded against the test's
204 fitness for purpose; these purposes are defined in the WOAHP Validation Template (chapter 1.1.6), which
205 identifies six main purposes for which diagnostic tests may be carried out. The table is intended ~~to be~~
206 as a general guide to test application; ~~the fact that a test is recommended does not necessarily mean~~
207 that a laboratory is competent to perform it. The laboratory quality system should incorporate provision
208 of evidence of competency.

209 In ~~the veterinary profession laboratories~~, other standard methods (published in international, regional,
210 or national standards) or fully validated methods (having undergone a full collaborative study and that
211 are published or issued by an authoritative technical body such as the AOAC International) may be
212 preferable to use, but ~~may not be~~ available. Many veterinary laboratories develop or modify methods,
213 and most laboratories have test systems that use non-standard methods, or a combination of standard

214 and non-standard methods. In veterinary laboratories, even with the use of standard methods, some in-
215 house evaluation, optimisation, or validation is generally must be done required to ensure valid results.

216 Customers and laboratory staff must have a clear understanding of the performance characteristics of
217 the test, and customers should be informed if the method is non-standard. Many veterinary testing
218 laboratories will therefore need to demonstrate competence in the development, adaptation, verification
219 and validation of test methods.

220 This *Terrestrial Manual* provides more detailed and specific guidance on test selection, optimisation,
221 standardisation, and validation in chapter 1.1.6. ~~Chapter 1.1.6 refers to chapters 2.2.1–2.2.8~~
222 ~~Recommendations for validation of diagnostic tests~~ that deal with the development and optimisation of
223 fundamentally different assays such as antibody, antigen and nucleic acid detections tests,
224 measurement uncertainty, statistical approaches to test validation, selection and use of reference
225 samples and panels, validation of diagnostic tests for wildlife, and comparability experiments after
226 changes in a validated test method.

227 The following are key test method issues for those involved in the quality management of the laboratory.

228 **7.1. Selection of the test method**

229 Valid results begin with the selection of a test method that meets the needs of the laboratory's
230 customers in addressing their specific requirements (fitness for purpose). Some issues relate
231 directly to the laboratory, others to the customer.

232 **7.1.1. Considerations for the selection of a test method**

- 233 i) International acceptance;
- 234 ii) Scientific acceptance;
- 235 iii) Appropriate or current technology;
- 236 iv) Suitable performance characteristics (e.g. analytical and diagnostic sensitivity and
237 specificity, repeatability, reproducibility, isolation rate, limits of detection, precision,
238 trueness, and uncertainty);
- 239 v) Suitability of the test in the species and population of interest;
- 240 vi) Sample type (e.g. serum, tissue, milk) and its expected quality or state on arrival at
241 the laboratory;
- 242 vii) Test target (e.g. antibody, antigen, live pathogen, nucleic acid sequence);
- 243 viii) Test turnaround time;
- 244 ix) Resources and time available for development, adaptation, evaluation;
- 245 x) Intended use (e.g. export, import, surveillance, screening, diagnostic, confirmatory);
- 246 xi) Safety factors and biocontainment requirements;
- 247 xii) Customer expectations;
- 248 xiii) ~~Throughput of test~~ Sample numbers and required throughput (automation, robot);
- 249 xiv) Cost of test, per sample;
- 250 xv) Availability of reference standards, reference materials and proficiency testing
251 schemes. (See also chapter 2.2.6.).

252 **7.2. Optimisation and standardisation of the test method**

253 Once the method has been selected, it must be set up at the laboratory. Additional optimisation
254 is necessary, whether the method was developed in-house (validation) or imported from an
255 outside source (verification). Optimisation establishes critical specifications and performance
256 standards for the test process as used in a specific laboratory.

257

7.2.1. Determinants of optimisation

- 258 i) Critical specifications for equipment, ~~instruments-consumables, and~~ reagents (e.g.
259 chemicals, biologicals), reference standards, reference materials, and internal
260 controls;
- 261 ii) Robustness – critical control points and acceptable ranges, attributes or behaviour
262 at critical control points, using statistically acceptable procedures;
- 263 iii) Quality control activities necessary to monitor critical control points;
- 264 iv) The type, number, range, frequency, and arrangement of test run controls;
- 265 v) Criteria for ~~non-subjective~~ objective acceptance or rejection of ~~a batch of~~ test results;
- 266 vi) Criteria for ~~the~~ interpretation and reporting of test results;
- 267 vii) ~~A Documented test method and reporting procedure for use by laboratory staff;~~
- 268 viii) Evidence of technical competence for those ~~who performing~~ the test processes
269 methods, authorising test results and interpreting results.

270

7.3. Validation of the test method

271 Test method validation evaluates the test for its ~~fitness for a given use purpose~~ by establishing
272 ~~test~~ performance characteristics such as sensitivity, specificity, and isolation rate; and diagnostic
273 parameters such as positive or negative cut-off, repeatability, reproducibility and titre of interest
274 or significance. Validation should be ~~done~~ performed using an optimised, documented, and fixed
275 procedure. The extent and depth of the validation process will depend on logistical and risk
276 factors. ~~It and~~ may involve any number of activities and amount of data, with subsequent data
277 analysis using appropriate statistical methods (Chapter 1.1.6.). Acknowledging diagnostic test
278 validation science as a key element in the effective detection of infectious diseases, WOAH
279 recently published a Special Issue representing an up-to-date compilation of the relevant
280 standards (WOAH and non-WOAH) and guidance documents for all stages of diagnostic test
281 validation and proficiency testing, including design and analysis, as well as clear, complete and
282 transparent reporting of validation studies in the peer-reviewed literature (Colling & Gardner,
283 2021). It is important to note that the current version of ISO 17025:2017 specifies that personnel
284 must be authorised to perform validation and related activities, which means that training in
285 validation and verification methods, including results interpretation, is likely to become more
286 important to prove competence (Colling & Gardner, 2021). It should also be noted that for
287 vetinary laboratories, limited availability of suitable material may render validation difficult;
288 under these circumstances it is necessary to highlight the limited validation status when
289 reporting results and their interpretation (Stevenson et al., 2021).

290

7.3.1. Activities that validation might include

- 291 i) Field or epidemiological studies, including disease outbreak investigations and
292 testing of samples from infected and non-infected animals;
- 293 ii) Development of testing algorithms for specific purposes, e.g. surveillance, outbreak
294 investigations, etc.;
- 295 iii) Repeat testing in the same laboratory to establish the effect of variables such as
296 operator, reagents, equipment;
- 297 iv) Comparison with other, preferably standard methods and with reference standards
298 (if available);
- 299 iii) Collaborative studies with other laboratories using the same documented method.
300 Ideally organised by a reference laboratory and including testing a panel of samples
301 of undisclosed composition or titre with expert evaluation of results and feedback to
302 ~~the~~ participants to estimate reproducibility;
- 303 iv) Reproduction of data from an accepted standard method, or from a ~~reputable peer-~~
304 reviewed publication (verification);

305 vii) Experimental infection ~~or disease outbreak studies;~~

306 viii) Analysis of internal quality control data.

307 vii) Field or epidemiological studies, including disease outbreak investigations and
308 testing of samples from infected and non-infected animals;

309 viii) Development of testing algorithms for specific purposes, e.g. surveillance, outbreak
310 investigations, etc.;

311 Validation is always a balance between cost, risk, and technical possibilities. There
312 may be cases where ~~quantities such as only basic~~ accuracy and precision can ~~only~~
313 ~~be given determined, e.g. when the disease is not present in a simplified way country~~
314 ~~or region~~. Criteria and procedures for the correlation of test results for diagnosis of
315 disease status or for regulatory action must be developed. The criteria and
316 procedures developed should account for screening methods, retesting and
317 confirmatory testing.

318 ~~Test validation is covered in chapter 1.1.6.~~

319 **7.4. Uncertainty of the test method**

320 Statistically relevant numbers of samples from infected and non-infected animals are discussed
321 in chapter 1.1.6. test validation and chapter 2.2.5 statistical approaches to validation.

322 **7.4. Estimation of Measurement Uncertainty**

323 Measurement of Uncertainty (MU) is “a parameter associated with the result of a measurement
324 that characterises the dispersion of values that could reasonably be attributed to the measure”
325 (Eurachem, 2012). Uncertainty of measurement does not imply doubt about a result but rather
326 increased confidence in its validity. It is not the equivalent to *error*, as it may be applied to all
327 test results derived from a particular procedure.

328 Laboratories must estimate the MU for each test method resulting in a quantitative measurement
329 included in their scope of accreditation, and for any methods used to calibrate equipment,
330 included in their scope of accreditation (ISO/IEC 17025, ~~2005-2017b~~).

331 Tests can be broadly divided into two groups: quantitative (e.g. biochemical assays, enzyme-
332 linked immunosorbent assays [ELISA], titrations, real-time polymerase chain reaction [PCR],
333 pathogen enumeration, etc.); and qualitative (bacterial culture, parasite identification, virus
334 isolation, endpoint PCR, immunofluorescence, etc.).

335 The determination of MU is well established in *quantitative* measurement sciences (ANSI,
336 1997). It may be given as a numeric expression of reliability and is commonly shown as a stated
337 range. Standard deviation (SD) and ~~confidence reference~~ interval (~~C-R~~) are examples of the
338 expression of MU, for example the optical density result of an ELISA expressed as $\pm n$ SD,
339 where n is usually 1, 2 or 3. The confidence interval (usually 95%) gives an estimated range in
340 which the result is likely to fall, calculated from a given set of test data. For quantitative
341 measurements, example for a top-down or control-sample approach are provided for an
342 antibody ELISA in chapter 2.2.4, and by the Australian government webpage⁴. An example for
343 a quantitative PCR hydrolysis probe (TaqMan) assay is provided by Newberry & Colling (2021).

344 The ISO/IEC 17025 requirement for “quality control procedures for monitoring the validity of
345 tests” implies that the laboratory must use quality control procedures that cover all major sources
346 of uncertainty. There is no requirement to cover each component separately. Laboratories may
347 establish acceptable specifications, criteria, ranges, etc., at critical control points for each
348 component of the test process. The laboratory can then implement appropriate quality control

⁴ Australian Government, Department of Agriculture, Fisheries and Forestry. Worked examples of measurement uncertainty. Measurement uncertainty in veterinary diagnostic testing – DAFF (agriculture.gov.au) (accessed 15 March 2023).

349 measures at these critical points, or seek to reduce or eliminate the uncertainty effect of each
350 component.

351 **7.4.1. Potential sources of uncertainty include:**

- 352 i) Sampling;
- 353 ii) Contamination;
- 354 iii) Sample transport and storage conditions;
- 355 iv) Sample processing;
- 356 v) Reagent quality, preparation and storage;
- 357 vi) Type of reference material;
- 358 vii) Volumetric and weight manipulations;
- 359 viii) Environmental conditions;
- 360 ix) Equipment effects;
- 361 x) Analyst or operator bias;
- 362 xi) Biological variability;
- 363 xii) Unknown or random effects.

364 Systematic errors or bias determined by validation must be corrected by changes in the
365 method, adjusted for mathematically, or have the bias noted as part of the report
366 statement.

367 If an adjustment is made to a test or procedure to reduce uncertainty or correct bias then
368 a new source of uncertainty is introduced (the uncertainty of the correction). This must be
369 assessed as part of the MU estimate.

370 The application of the principles of MU to *qualitative* testing is less well defined. The
371 determination and expression of MU has not been standardised for veterinary (or medical,
372 food, or environmental) testing laboratories, but sound guidance exists and as
373 accreditation becomes more important, applications are being developed. The ISO/IEC
374 17025 standard recognises that some test methods may preclude metrologically and
375 statistically valid calculation of uncertainty of measurement. In such cases the laboratory
376 must attempt to identify and estimate all the components of uncertainty based on
377 knowledge of the performance of the method and making use of previous experience,
378 validation data, internal control results, etc.

379 Many technical organisations and accreditation bodies (e.g. AOAC International, ISO,
380 NATA, A2LA, Standards Council of Canada, UKAS, Eurachem, the Cooperation of
381 International Traceability in Analytical Chemistry) teach courses or provide guidance on
382 MU for laboratories seeking accreditation.

383 ~~The ISO/IEC 17025 requirement for “quality control procedures for monitoring the validity~~
384 ~~of tests” implies that the laboratory must use quality control procedures that cover all~~
385 ~~major sources of uncertainty. There is no requirement to cover each component~~
386 ~~separately. Laboratories may establish acceptable specifications, criteria, ranges, etc., at~~
387 ~~critical control points for each component of the test process. The laboratory can then~~
388 ~~implement appropriate quality control measures at these critical points, or seek to reduce~~
389 ~~or eliminate the uncertainty effect of each component. Measurement Uncertainty is~~
390 ~~covered in chapter 2.2.4.~~

391 ~~**7.4.1. Components of tests with sources of uncertainty include:**~~

- 392 ~~i) Sampling;~~
- 393 ~~ii) Contamination;~~

- 394 iii) ~~Sample transport and storage conditions;~~
395 iv) ~~Sample processing;~~
396 v) ~~Reagent quality, preparation and storage;~~
397 vi) ~~Type of reference material;~~
398 vii) ~~Volumetric and weight manipulations;~~
399 viii) ~~Environmental conditions;~~
400 ix) ~~Equipment effects;~~
401 x) ~~Analyst or operator bias;~~
402 xi) ~~Biological variability;~~
403 xii) ~~Unknown or random effects.~~

404 ~~Systematic errors or bias determined by validation must be corrected by changes in the~~
405 ~~method, adjusted for mathematically, or have the bias noted as part of the report~~
406 ~~statement.~~

407 ~~If an adjustment is made to a test or procedure to reduce uncertainty or correct bias then~~
408 ~~a new source of uncertainty is introduced (the uncertainty of the correction). This must be~~
409 ~~assessed as part of the MU estimate.~~

410 Additional information on the analysis of uncertainty may be found in the Eurachem
411 Guides to Quantifying Uncertainty in Measurement (Eurachem, 2012) and Use of
412 ~~uncertainty information in compliance assessment~~ Uncertainty Information in Compliance
413 Assessment (Eurachem, 2007).

414 **7.5. Implementation and use of the test method**

415 Training should be a planned and structured activity with steps to ensure adequate supervision
416 is maintained while analysts are being trained. Depending on the complexity of the test and the
417 experience of the analyst, training may include any combination of reading and understanding
418 the documented test method, initial demonstration, performance of the test under supervision
419 and independent performance. Analysts should ~~be able to demonstrate~~ proficiency in using the
420 test method prior to ~~producing~~ being authorised to produce reported results, and on an ongoing
421 basis.

422 The laboratory must be able to demonstrate traceability for all accredited tests and the principle
423 should apply to all tests whether accredited or not. This covers all activities relating to test
424 selection, development, optimisation, standardisation, validation, verification, implementation,
425 reporting, personnel, quality control and quality assurance (see also Section 7.3.1. point vi).
426 Traceability is achieved by using appropriate documented project management, record keeping,
427 data management and archiving systems.

428 **8. Strategic planning**

429 Laboratories should have evidence of continual improvement, which is an obligatory requirement
430 for accredited laboratories. The laboratory must ~~be knowledgeable of and stay~~ maintain current
431 ~~with knowledge of~~ the relevant quality and technical ~~management~~ standards and with methods
432 used to demonstrate laboratory competence and establish and maintain technical validity. Evidence
433 of this may ~~be provided by~~ include:

- 434 i) Attendance at conferences, organisation of in-house or external meetings on diagnostics and
435 quality management;
436 ii) ~~Participation in~~ Membership of local and international organisations;
437 iii) ~~Participation in writing~~ Contribution to national and international standards (e.g. on ILAC and
438 ISO committees);

- 439 iv) Maintenance of current awareness of publications, writing through review of and reviewing
440 publications about diagnostic methods contribution to relevant literature;
- 441 v) Participation in training programmes, including visits to other laboratories;
- 442 vi) Conducting research;
- 443 vii) Participation in cooperative programmes (e.g. Inter-American Institute for Cooperation in
444 Agriculture);
- 445 viii) Exchange of procedures, methods, reagents, samples, personnel, and ideas;
- 446 ix) Planned, continual professional development and technical training;
- 447 x) Management reviews;
- 448 xi) Analysis of customer feedback;
- 449 xii) Root cause analysis of anomalies and implementation of corrective, preventive and
450 improvement actions, as well as effectiveness reviews.

451

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485 NB: FIRST ADOPTED IN 1996 AS *GOOD LABORATORY PRACTICE, QUALITY CONTROL AND QUALITY ASSURANCE*.
486 MOST RECENT UPDATES ADOPTED IN 2017.

Annexe 5. Chapter 1.1.9. 'Tests for sterility and freedom from contamination of biological materials intended for veterinary use'

CHAPTER 1.1.9.

TESTS FOR STERILITY AND FREEDOM FROM CONTAMINATION OF BIOLOGICAL MATERIALS INTENDED FOR VETERINARY USE

INTRODUCTION

The international trade-related movements of biological materials intended for veterinary use are subject to restrictions imposed to minimise the spread of animal and human pathogens. Countries may impose requirements for proof-of-freedom testing before allowing the regulated importation of materials of animal derivation and substances containing such derivatives. Where chemical or physical treatments are inappropriate or inefficient, or where evidence is lacking of the effectiveness of the treatment is lacking, there may be general or specific testing requirements imposed by authorities of countries receiving such materials. This chapter provides guidance on the approach to such regulated testing, particularly as might be applied to the movement of vaccine master seed and master cell stocks, and to related biological materials used in manufacturing processes. The term seed stocks is used when testing live products, for killed products the preferred reference is master cell stocks. While the onus for ensuring safety of a product remains with the manufacturer and may be regulated by therapeutic guidelines, this chapter provides procedures that are designed in particular to minimise the risk of undetected contaminants in veterinary therapeutics and biological reagents causing the cross-border spread of agents of concern to particular importing countries. In their review "Extraneous agent detection in vaccines" Farsang & Kulcsar, 2012 reported the following examples of contamination of vaccines with extraneous agents: a) Foamy virus (Spumaretroviridae) was identified as a contaminant of primary monkey kidney cultures used for vaccine production in the early 1950, b) In the 1960s it was shown that yellow fever live attenuated vaccines prepared in chicken embryo fibroblasts were infected with avian leukosis virus (ALV). c) Calicivirus was found in Chinese hamster ovary (CHO) cells, d) Newcastle disease vaccine strains were found in different live poultry vaccines, e) in 1990 a live attenuated multi component canine vaccine was contaminated with a serotype of Bluetongue virus causing abortions and death in pregnant bitches, f) Fetal calf serum transmitted Pestiviruses (BVDV types 1 and 2) are one of the most common extraneous agents in veterinary and human vaccines, g) RD114 is a replication-competent feline endogenous gamma retrovirus which contaminated canine corona and parvovirus vaccines, h) a notable case of human vaccine contamination may have been when in the 20th century tens of millions of people worldwide were immunised with polio vaccines containing simian virus 40 (SV40). SV40 was found to cause cancer in animals and is associated with human brain, bone and lung cancers, however, a clear connection was not found between this certain vaccine and any human tumour case, i) a porcine circovirus 1 (PCV1) was found in a rotavirus vaccine widely used worldwide for children. Farsang & Kulcsar (2012) and WHO (2015) describe case studies of veterinary and human vaccines contaminated

41 with extraneous agents and findings support the need of accurate and validated
42 amplification and detection methods as key elements for effective detection and control.
43 Further examples are given in Section G. Protocol examples below. Control of
44 contamination with transmissible spongiform encephalopathy (TSE) agents is not covered
45 in this chapter because standard testing and physical treatments cannot be used to ensure
46 freedom from these agents. Detection methods are described in Chapter 3.4.5. Bovine
47 spongiform encephalopathy.

48 Sterility is defined as the absence of viable microorganisms, which for the purpose of this
49 chapter, includes viruses. It should be achieved using aseptic techniques and validated
50 sterilisation methods, including heating, filtration, chemical treatments, and irradiation that
51 fits the intended purpose. Freedom from contamination is defined as the absence of
52 specified viable microorganisms. This may be achieved by selecting materials from sources
53 shown to be free from specified microorganisms and by conducting subsequent procedures
54 aseptically. Adequate assurance of sterility and freedom from contaminating
55 microorganisms can only be achieved by proper control of the primary materials used and
56 their subsequent processing. Tests on intermediate products are necessary throughout the
57 production process to check that this control has been achieved.

58 Biological materials subject to contamination that cannot be sterilised before or during use
59 in vaccine production, such as ingredients of animal origin, e.g. serum and trypsin, primary
60 and continuous ~~cells and~~ cell lines, and viral or bacterial seed stocks, etc., should be tested
61 for viable extraneous agents before use. Assays to detect viral contaminants, if present,
62 can be achieved by various culture methods, including use of embryonated eggs, which
63 are supported by cytopathic effects (CPE) detection/embryo death, fluorescent antibody
64 techniques and ~~other suitable~~ (fit for purpose), methods such as polymerase chain reaction
65 (PCR) and antigen detection ELISA (enzyme-linked immunosorbent assay). As is
66 explained in more detail in this chapter care must be taken when using PCR and ELISA
67 techniques for detection as such tests do not distinguish viable from non-viable agent
68 detection. Specific assays to detect other contaminants, such as fungi, protozoa and
69 bacteria (including rickettsia and mycoplasma) are also described.

70 ~~Avian materials and vaccines are required to be inoculated on to primary avian cell cultures~~
71 ~~or eggs for the detection of avian viruses. A combination of general tests, for example to~~
72 ~~detect haemadsorbing, haemagglutinating and CPE-causing viruses and specific~~
73 ~~procedures aimed at the growth and detection of specific viruses is recommended to~~
74 ~~increase the probability of detection. Assays to detect other contaminants, such as bacteria,~~
75 ~~fungi, protozoa, rickettsia and mycoplasma are also described.~~

76 ~~Procedures applied~~ Testing procedures should be validated and found to be “fit for
77 purpose” following Chapter 1.1.6. Validation of diagnostic assays for infectious diseases of
78 terrestrial animals, where possible.

79 It is a requirement of many regulators, that a laboratory testing report notes the use of
80 validated procedures and describes the validated procedures in detail including acceptance
81 criteria. This gives the regulator transparency in the procedures used in a testing laboratory.

82 The validation assessment of an amplification process in cell culture should include
83 documentation of the history of permissive cell lines used, reference positive controls and
84 culture media products used in the process of excluding adventitious agents, to ensure the
85 process is sound and is not compromised. The validation assessment should give
86 information (published or in-house) of the limitations that may affect test outcomes and an
87 assessment of performance characteristics such as analytical specificity and sensitivity of
88 each cell culture system, using well characterised, reference positive controls.

89 It is the responsibility of the submitter to ~~assure~~ ensure a representative selection and
90 number of items to be tested. ~~The principles of~~ Appendix 1.1.2.1 Epidemiological
91 approaches to sampling: sample size calculations of Chapter 1.1.2 Collection, submission
92 and storage of diagnostic specimens ~~apply~~ describes the principles to be applied. Adequate

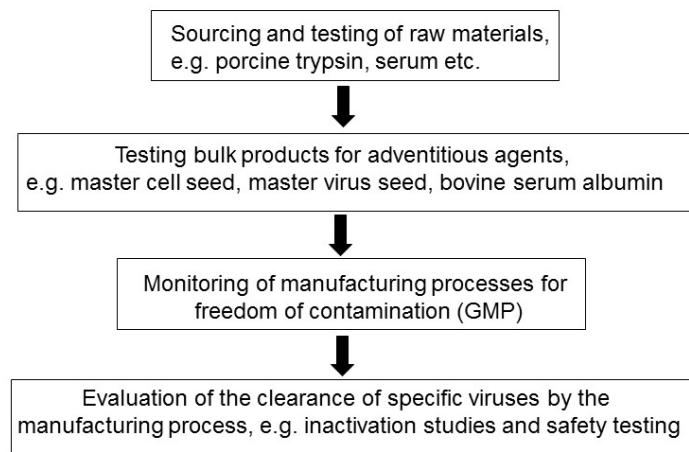
93 ~~transportation is described in Chapter 1.1.2 and Chapter 1.1.3~~ Transport of biological
94 materials describe transportation requirements.

95 A. AN OVERVIEW OF TESTING APPROACHES

96 Although testing is seen as a key component of biosafety in biological products intended for veterinary use,
97 testing is not enough to ensure a given product is free of viable infectious contaminants, and so a holistic,
98 multifaceted approach must be taken. Such an approach includes risk assessment, risk mitigation and
99 management strategies (Barone *et al.*, 2020). In general:

- 100 • Primary materials must be collected from sources shown to be free from contamination and handled in
101 such a way as to minimise contamination and the opportunities for any contaminants to multiply (Figure
102 1).
- 103 • Materials that are not sterilised and those that are to be processed further after sterilisation must be
104 handled aseptically. Such materials will require further assessment of freedom of contaminants at certain
105 stages of production to assure freedom of adventitious agents.
- 106 • Materials that can be sterilised without their biological activities being affected unduly must be sterilised
107 by a method effective for the pathogens ~~concerned of concern~~. The method must reduce the level of
108 contamination to be undetectable, as determined by an appropriate sterility test study. ~~(See Section D.1.~~
109 ~~below)~~. If a sterilisation process is used, it shall be validated to demonstrate that it is fit for purpose.
110 Suitable controls will be included in each sterilisation process to monitor efficiency.
- 111 • The environment in which any aseptic handling is carried out must be maintained in a clean state,
112 protected from external sources of contamination, and controlled to prevent internal contamination. Rules
113 governing aseptic preparation of vaccines are documented in Chapter 2.3.3 *Minimum requirements for*
114 *the organisation and management of a vaccine manufacturing facility*.

115 **Figure 1. Testing algorithm Risk assessment flowchart for vaccine production.**



116

117 Some procedures have been properly validated and found to be “fit for purpose”, whilst others may have
118 undergone only limited validation studies. For example, methods for bacterial and fungal sterility may have
119 not been formally validated although they have been used for many years. In particular, ~~the~~ *in-vivo* and ~~cell~~
120 ~~culture~~ *in-vitro* methods have essentially unknown sensitivity and specificity (Sheets *et al.*, 2012) though
121 there is an accepted theoretical sensitivity, regarding cell culture of 1 ~~colony~~ plaque-forming unit (CFU
122 PFU). For example, an evaluation of methods to detect bovine and porcine viruses in serum and trypsin
123 based on United States (of America) Code of Federal Regulations, Title 9 (9CFR) revealed gaps in
124 sensitivity, even within virus families (Marcus-Secura *et al.*, 2011). It is therefore important to interpret, and
125 report results in the light of specific conditions of cultures employed and considering sensitivity and
126 specificity of detection systems.

127 Newer, more sensitive methods such as molecular assays may afford the ability to detect contaminants,
128 which may not be successfully amplified in traditional culturing systems. The detection range can be
129 broadened by using family specific primers and probes if designed appropriately. However, most, if not all
130 ~~such new molecular-based~~ tests are also able to detect evidence for non-infectious contaminants, such as
131 traces of nucleic acid from inactivated contaminants. ~~Follow up testing would be required to determine the~~
132 ~~nature of the contaminant, for example, non-infectious nucleic acid or infectious virus. Attempts at virus~~
133 ~~isolation or sequencing may remedy this.~~ Note: molecular assays if not designed as fit for purpose may
134 miss detection of contaminating agents or lack sensitivity to do so (Hodinka, 2013).

135 More recently metagenomic high throughput sequencing (HTS) workflows have shown potential for quality
136 control of biological products (van Borm *et al.*, 2013) and vaccines (Baylis *et al.*, 2011; Farsang & Kulcsar,
137 2012; Neverov & Chumakov, 2010; Onions & Kolman, 2010; Victoria *et al.*, 2010) in particular for the
138 identification and characterisation of unexpected highly divergent pathogen variants (Miller *et al.*, 2010;
139 Rosseel *et al.*, 2011) that may remain undetected using targeted diagnostic tests. Nevertheless, targeted
140 assays, e.g. amplification in cell culture followed by polymerase chain reaction (PCR) may be superior to
141 HTS for specific agent detection (Wang *et al.*, 2014) due to lack of sensitivity of HTS at this time. Chapter
142 1.1.7. gives an overview of the standards for high throughput sequencing, bioinformatics and computational
143 genomics. Similarly, recent improvements in protein and peptide separation efficiencies and highly accurate
144 mass spectrometry have promoted the identification and quantification of proteins in a given sample. Most
145 of these new technologies are broad screening tools, limited by the fact that they cannot distinguish
146 between viable and non-viable organisms.

147 Given the availability of new technologies, there will be future opportunities and challenges to determine
148 presence of extraneous agents in biologicals intended for veterinary use for industry and regulators.
149 Problems can arise when the presence of genome positive results are interpreted as evidence for the
150 presence of contamination (Mackay & Kriz, 2010). When using molecular technologies, it is important to
151 understand the correlation between genome detection and detection of live virus agent. It cannot be
152 assumed that detection of genome corresponds to the presence of an infectious agent.

153 **B. LIVING VIRAL VACCINES FOR ADMINISTRATION BY INJECTION, OR THROUGH** 154 **DRINKING WATER, SPRAY, OR SKIN SCARIFICATION**

- 155 1. Materials of animal origin ~~shall should~~ be ~~(a) sterilised, or (b) and~~ obtained from healthy animals that, in
156 so far as is possible, should be shown to be free from pathogens that can be transmitted from the species
157 of origin to the species to be vaccinated, or any species in contact with them by means of extraneous
158 agents testing.
- 159 2. Seed lots of virus, any continuous cell line and biologicals used for virus growth ~~shall should~~ be shown to
160 be free from ~~viable~~ bacteria, fungi, mycoplasmas, protozoa, rickettsia, ~~and~~ extraneous viruses ~~and other~~
161 ~~pathogens~~ that can be transmitted from the species of origin to the species to be vaccinated or any species
162 in contact with them. ~~There may be some exceptions for a limited number of non-pathogenic bacteria and~~
163 ~~fungi to be present in live viral vaccines produced in eggs and administered through drinking water, spray,~~
164 ~~or skin scarification.~~

165 For ~~the~~ production of vaccines in embryonated chicken eggs and the quality control procedures for these
166 vaccines, it is recommended ~~(required in many countries)~~ that eggs from specific pathogen-free birds
167 should be used.

- 168 3. Each batch of vaccine ~~shall should~~ pass tests for freedom from extraneous agents that are consistent
169 with the importing country's requirements for accepting the vaccine for use. Some examples of published
170 methods that document acceptable testing procedures-processes in various countries include: (US) Code
171 of Federal Regulations (2015); European Pharmacopoeia (2014); European Commission (2006); World
172 Health Organization (WHO) (1998; 2012) and Department of Agriculture (of Australia) (2013).
 - 173 • Code of Federal Regulations, Title 9 (9CFR) (of the United States of America) (2015).
 - 174 • Department of Agriculture, Forest and Fisheries (Australia) (2013).
 - 175 • Department of Agriculture, Forest and Fisheries (Australia) (2021b) Live Veterinary Vaccines.
 - 176 • Regulation on Veterinary Drug Administration (China [People's Republic of]) (2020).
 - 177 • European Medicines Agency Sciences Medicines Health (2016).

- 178 • European Pharmacopoeia, 10th Edition (2021).
179 • World Health Organization (WHO) (1998; 2012).

180 4. Tests for ~~sterility~~ freedom of contamination ~~shall~~ should be appropriate to prove that the vaccine is free
181 from viable extraneous viruses, bacteria including rickettsia and mycoplasmas, fungi, and protozoa. Each
182 country will have ~~particular~~ requirements as to what agents are ~~necessary to exclude~~ should be tested for
183 and ~~what by which~~ procedures are ~~acceptable~~. Such tests will include amplification of ~~viable~~ extraneous
184 agents using cell culture that is susceptible to ~~particular~~ known viruses of the species of concern, tests in
185 embryonated eggs, bacterial, mycoplasma and fungal culturing techniques and, where ~~necessary and~~
186 ~~possible there is no alternative~~ ie, tests involving animal inoculation. PCR, fluorescence antibody test
187 (FAT), presence of colonies or cytopathic effects (CPE) and antigen detection ELISA ~~will~~ can be used for
188 detection purposes after amplification using culturing techniques to improve specificity and sensitivity. If
189 *in-vitro* or *in-vivo* amplification of the target agent is not possible, direct PCR may be useful if validated
190 for this purpose.

191 **C. LIVING VIRAL VACCINES FOR ADMINISTRATION THROUGH DRINKING WATER,** 192 **SPRAY, OR SKIN SCARIFICATION**

193 ~~1. Section B applies.~~

194 ~~2. A limited number of contaminating, non-pathogenic bacteria and fungi may be permitted (see Section~~
195 ~~1.2.2 General Procedure for testing live viral vaccines produced in eggs and administered through drinking~~
196 ~~water, spray, or skin scarification for the presence of bacteria and fungi).~~

197 **D.C. INACTIVATED VIRAL AND BACTERIAL VACCINES**

198 1. Each batch of vaccine shall pass a test for inactivation of the vaccinal ~~virus seed~~ and should include
199 inactivation studies on representative extraneous agents if the virus or bacterial seed has not already
200 been tested and shown to be free from extraneous agents. An example of a simple inactivation study
201 could include assessment of the titre of live vaccine before and after inactivation and assessing the log₁₀
202 drop in titre during the inactivation process. This would give an indication of the efficacy of the inactivation
203 process. There is evidence that ~~virus~~ titration tests may not have sufficient sensitivity to ensure complete
204 inactivation. In these circumstances, a specific innocuity test would need to be developed and validated
205 to be fit for increased sensitivity. To increase sensitivity more than one passage would be required
206 depending on the virus or bacteria of concern. An example of this approach can be found at:
207 https://www.aphis.usda.gov/animal_health/vet_biologics/publications/memo_800_117.pdf (accessed 25
208 July 2023).

209 2. If studies on representative extraneous agents are required, then spiking inactivated vaccine with live
210 representative agents and following the example of an inactivation study ~~as in D.1 above~~ would ~~would~~ could be
211 useful. The inactivation process and the tests used to detect live ~~virus agent~~ after inactivation must be
212 validated and shown to be suitable for their intended purpose.

213 In addition, each country may have ~~particular~~ its own requirements for sourcing or tests for sterility as
214 detailed in Section B above.

215 **E. D. LIVING BACTERIAL VACCINES**

216 1. See Section B applies.

217 2. Seed lots of bacteria shall be shown to be free from other bacteria as well as fungi and mycoplasmas,
218 protozoa, rickettsia, and extraneous viruses. Agents required for exclusion will be dependent on the
219 country accepting the vaccine for use. Use of antibiotics to 'inactivate' the living bacterial seed or vaccine
220 prior to exclusion of viruses and fungi is recommended to ensure testing in culture is sensitive.

221 Interference testing is recommended to ensure that the antibiotics used do not affect the growth of the
222 extraneous virus or fungi that is being excluded. Sonication may also be useful

223 Interference testing is required to ensure that antibiotics used (or sonication) does not affect the growth
224 of extraneous virus or fungi being excluded, compromising the test outcome.

225 Due to the difficulties and reduced sensitivity in exclusion of extraneous bacteria and some mycoplasma,
226 protozoa, and rickettsia from high-titred seed lots of bacteria, the use of narrow-range antibiotics aimed
227 specifically at reducing seed lot bacteria is ~~recommended~~ useful if antibiotics do not affect the growth of
228 bacteria being excluded. The optimal concentration of antibiotics can be determined in a dilution
229 experiment such as documented in 9CFR Section 113.25(d). Other methods of exclusion of extraneous
230 bacteria from bacterial seeds may include filtering for size exclusion such as removing bacteria seed to
231 look for mycoplasma contamination and use of selective culturing media. Such processes would require
232 ~~validation~~ verification to ensure the process does not affect the sensitivity of exclusion of extraneous
233 agents of concern.

234 3. Sonication of a living bacterial seed may be useful when excluding specific viral agents. Once again, the
235 inactivation procedure would require a verification process to ensure the adventitious virus being excluded
236 is not affected by the treatment. Use of a suitable reference virus control during the exclusion process
237 would be required.

238 4. Direct PCR techniques may be useful when culturing processes fail to be ~~sensitive~~ successful in detecting
239 extraneous bacteria from live bacterial seeds or vaccines.

240 **~~F. INACTIVATED BACTERIAL VACCINES~~**

241 1. ~~Section D applies. It should not be necessary to test for extraneous viruses that would not grow in~~
242 ~~bacteriological culture media as long as freedom from contamination of all starting materials can be~~
243 ~~assured. Complete inactivation of the vaccinal bacteria should be demonstrated by means of titration and~~
244 ~~innocuity tests — in some cases general bacterial sterility testing (Section I.2.1) may suffice.~~

245 **G-E. SERA, PLASMA AND DIAGNOSTIC AGENTS FOR ADMINISTRATION TO** 246 **ANIMALS**

247 1. Section B-4 applies for sera/diagnostic agents that are not inactivated. Section C applies for non-
248 inactivated sera/diagnostic agents.

249 2. Some countries require quarantine, health certification, and tests for specific diseases to be completed
250 for all serum and plasma donor animals, for example, 9CFR (2015) and Australian Quarantine Policy and
251 Requirements for the Importation of Live and Novel Veterinary Bulk and Finished Vaccines (1999). For
252 some diseases, for example equine infectious anaemia, the product (plasma) must be stored until the
253 seroconversion period has been exceeded and the donors tested negative.

254 3. ~~It is recommended that each batch of non-inactivated serum be assessed for viable extraneous agents,~~
255 ~~including mycoplasma. Each batch of serum shall pass a test for freedom from extraneous agents.~~
256 ~~Suitable test methods have been published for various countries, for example, European Pharmacopoeia~~
257 ~~(2014); 9 CFR (2015) and Australian Quarantine Policy and Requirements for the Importation of Live and~~
258 ~~Novel Veterinary Bulk and Finished Vaccines (1999) and Department of Agriculture (of Australia) (2013).~~

259 ~~4. Inactivated serum, Section D applies.~~

260 5. ~~Section B or D may apply if a virus is used in the production of the diagnostic agent; Section E or F may~~
261 ~~apply if a bacterium is used.~~

H. F. EMBRYOS, OVA, SEMEN

Special precautions must be taken with relation to the use of embryos, ova, semen (Hare, 1985). Most countries will have regulatory guidelines for import of these biologicals for veterinary use. Such guidelines can be found at various websites such as the European Commission (2015), FAO and Department of Agriculture Forest and Fisheries (2021a; 2021b), though many such some guidelines may give more detail in regard to the food safety aspect.

J. G. PROTOCOL EXAMPLES

1. ~~General procedures~~ Introduction to protocol examples

This section provides some examples to illustrate scope and limitations of testing protocols. It is not intended to be prescriptive or exhaustive. Examples are based on standards and published methods to increase the sensitivity for exclusion of live adventitious agents, using general and specific techniques.

In principle, proposed testing represents an attempted isolation of viable agents in culturing systems normally considered supportive of the growth of each specified agent or group of general agents. After amplification, potential pathogens can be detected further, by sensitive and specific diagnostic tests such as FAT or PCR if as required. General detection systems can include haemabsorbance and CPE by immunohistochemistry staining methods. The example procedures for sterility detection of contamination testing and general detection of viable virus, fungi, protozoa and bacteria (including rickettsia and mycoplasma, fungi and viruses) described below are derived from standards such as the 9CFR (2015), European Pharmacopoeia, (2014) 10th Edition (2021), European Commission (2006), WHO Medicines Agency Sciences Medicines Health (2016), Department of Agriculture, Forest and Fisheries (Australia) (2013) and World Health Organization (1998; 2012).

Individual countries or regions should adopt a holistic, risk-based approach to determine the appropriate testing protocols based on their animal health status. As well as applying general testing procedures documented in national or regional standards as mentioned above, it may be necessary to apply rigorous exclusion testing for specific agents that are exotic to the ~~particular~~ country or region of concern.

General procedures ~~will do~~ not necessarily detect all extraneous agents that may be present in biological material; however, they are useful as screening tests. Some examples of agents that may require specific methods for detection in biologicals refer to Table 1 below. Procedures documented in the Review of Published Tests to Detect Pathogens in Veterinary Vaccines Intended for Importation into Australia (2013) available from the Department of Agriculture, Forest and Water Resources, Australia Fisheries are able to address such agents in offering sensitive testing approaches based on reputable publications. A CVMP reflection paper published written by the European Medicines Agency Sciences Medicines Health Committee of Veterinary Medicinal Products (CVMP) in (2016), adopted in May 2017, documents lists specific test method approaches for a number of agents, listed in Table 1, that cannot be excluded using general test procedures (Table 1).

Exclusion of specific agents requires procedures that maximise sensitivity by providing ideal amplification and detection of the pathogen in question. Extraneous agents, for example, Maedi Visna virus, ~~bovine immunodeficiency virus,~~ (and other retroviruses), *Trypanosoma evansi* and porcine respiratory coronavirus are difficult to culture even using the most sensitive approaches. In these circumstances, application of molecular assays directly to the biological material ~~in question to assess,~~ assessing for the presence of nucleic acid from adventitious agents offers an alternative. ~~Refer to Table 1. Consideration must be noted as described in Section A.6 as,~~ though detection of the presence of non-viable and host associated agents may is also be detected using this procedure possible.

Table 1 gives examples of causative infectious agents that may be present in animal biologicals intended for veterinary use, for example PCV-1 in a rotavirus vaccine (WHO, 2015). BVDV is well known for its presence in many bovine associated biologicals, including cell culture. More recently, non-CPE pestivirus, BVD type 3 (HoBi-like) are found in foetal calf serum and cell culture. Classical Swine fever has contaminated various porcine cell lines used for African swine fever and FMDV diagnosis, and thus the potential for contamination of porcine based vaccines. PEDV is linked to spray-dried porcine plasma used for feed. This is not an

310 exhaustive list of agents of concern or by any means required for exclusion by every country based on risk,
 311 they are ~~just~~ examples of infectious agents that are not culturable using general culturing procedures and
 312 require ~~a more use of specialised culturing processes and~~ specific detection ~~process by means of the indirect~~
 313 ~~fluorescent antibody test, PCR or ELISA, where applicable processes~~. Notably, some subtypes of an agent
 314 type may be detectable by general methods, and some may require specialised testing for detection. For
 315 example, bovine adenovirus subgroup 1 (serotypes 1, 2, 3 and 9) can be readily isolated using general
 316 methods (Vero cells) however bovine adenovirus subgroup 2 (serotypes 4, 5, 6, 7, 8 and 10) are not readily
 317 isolated and required specialised methods for isolation.

318 **Table 1. ~~Some~~ Examples of infectious agents of veterinary importance**
 319 **that require ~~specialist~~ specialised culturing and detection techniques**

Rotaviruses	Pestiviruses (non-CPE)	Turkey rhinotracheitis
Porcine epidemic diarrhoea virus	Bluetongue virus	<i>Brucella abortus</i>
Porcine circoviruses (PCV 1, 2)	Swine pox virus	Rickettsias
Swine/equine influenza, some strains	Some adenoviruses	Protozoa
Bovine respiratory syncytial virus	<u>Rhabdoviruses (e.g. rabies virus)</u>	Some fungi (e.g. <i>Histoplasma</i>)

320 2. Example of detection of bacteria and fungi contamination

321 2.1. General procedure for assessing the sterility of viable bacteria and fungi

322 Standard tests for detecting extraneous bacteria and fungi (sterility testing) in raw materials, master
 323 cell stocks, or final product are the membrane filtration test or the direct inoculation sterility test.

324 For the membrane filtration technique, a filter having a nominal pore size not greater than 0.45 µm
 325 and a diameter of at least 47 mm should be used. Cellulose nitrate filters should be used if the
 326 material is aqueous or oily; cellulose acetate filters should be used if the material is strongly alcoholic,
 327 oily or oil-adjuvanted. Immediately before the contents of the container or containers to be tested are
 328 filtered, the filter is moistened with 20–25 ml of Diluent A or B.

329 2.1.1. Diluent A

330 Diluent A is for aqueous products or materials. Dissolve 1 g peptic digest of animal tissue in
 331 water to make 1 litre, filter, or centrifuge to clarify, adjust the pH to 7.1 ± 0.2, dispense into
 332 containers in 100 ml quantities, and sterilise by steam.

333 2.1.2. Diluent B

334 Diluent B is for oil-adjuvanted products or materials: Add 1 ml polysorbate 80 to 1 litre Diluent
 335 A, adjust the pH to 7.1 ± 0.2, dispense into containers in 100 ml quantities, and sterilise by
 336 steam.

337 If the biological being tested has antimicrobial properties, the membrane is washed three times after
 338 sample application with approximately 100 ml of the appropriate diluent (A or B). The membrane is
 339 then transferred ~~whole~~ to culture media, aseptically cut into equal parts and placed in media, or the
 340 media is transferred to the membrane in the filter apparatus. If the test sample contains merthiolate
 341 as a preservative, fluid thioglycolate medium (FTM) is used and the membranes are incubated at
 342 both 30–35°C and 20–25°C. If the test sample is a killed biological without merthiolate preservative,
 343 FTM is used at 30–35°C and soybean casein digest medium (SCDM) at 20–25°C. If the sample
 344 tested is a live viral biological, SCDM is used at both incubation temperatures. It has been suggested
 345 that sulfite-polymyxin-sulfadiazine agar be used to enhance the detection of *Clostridium* spp. when
 346 the membrane filtration technique is used (Tellez *et al.*, 2005).

347 If direct inoculation of culture media is chosen, a sterile pipette or syringe and needle are used to
 348 aseptically transfer the biological material directly into liquid media. If the biological being tested has

349 antimicrobial properties, the ratio of the inoculum to the volume of culture medium must be
 350 determined before the test is started, for example as explained in 9CFR 113.25(d) and detailed
 351 testing procedures can be found for example in supplemental assay method USDA SAM 903
 352 https://www.aphis.usda.gov/animal_health/vet_biologics/publications/sam903.pdf (accessed 24 July
 353 2023) (~~SAM) 903 USDA SAM 903, See~~
 354 ~~https://www.aphis.usda.gov/animal_health/vet_biologics/publications (accessed 4 July 2022).~~ To
 355 determine the correct medium volume to negate antimicrobial activity, 100 CFU of the control
 356 microorganisms listed in Table 2 are used. If the test sample contains merthiolate as a preservative,
 357 FTM is used in test vessels incubated at both 30–35°C and 20–25°C. Growth should be clearly visible
 358 after an appropriate incubation time (see Section 1.2.1.3 *Growth promotion and test interference*). If
 359 the test sample is a killed biological without merthiolate, or a live bacterial biological, FTM is used at
 360 30–35°C and SCDM at 20–25°C. If the test sample is a live viral biological, SCDM is used at both
 361 incubation temperatures. If the inactivated bacterial vaccine is a clostridial biological, or contains a
 362 clostridial component, the use of FTM with 0.5% added beef extract (FTMB) in place of FTM is
 363 preferred. It may also be desirable to use both FTM and SCDM for all tests.

364 **Table 2. Some American Type Culture Collection¹ strains with their respective**
 365 **medium and incubation conditions**

Medium	Test microorganism	Incubation	
		Temperature (°C)	Conditions
FTM	<i>Bacillus subtilis</i> ATCC # 6633	30–35	Aerobic
FTM	<i>Candida krusei</i> ATCC # 6258	20–25	Aerobic
SCDM	<i>Bacillus subtilis</i> ATCC # 6633	30–35	Aerobic
SCDM	<i>Candida krusei</i> ATCC # 6258	20–25	Aerobic
FTMB	<i>Clostridium sporogenes</i> ATCC # 11437	30–35	Anaerobic
FTMB	<i>Staphylococcus aureus</i> ATCC #6538	30–35	Aerobic

366 For both membrane filtration and direct inoculation sterility tests, all media are incubated for no fewer
 367 than 14 days. At intervals during incubation, and after 14 days' incubation, the test vessels are
 368 examined for evidence of microbial growth. Microbial growth should be confirmed by subculture and
 369 Gram stain.

370 **2.1.3. Example of growth promotion and test interference**

371 The sterility of the media should be confirmed by incubating representative containers at the
 372 appropriate temperature for the length of time specified for each test.

373 The ability of the culture media to support growth in the presence and absence of product,
 374 product components, cells, seeds, or other test material should be validated for each product
 375 to be tested, and for each new batch or lot of culture media for example as outlined in 9CFR
 376 113.25(b). Detailed testing procedures can be found for example in USDA SAMs 900-902,
 377 See USDA APHIS | Supplemental Assay Methods - 900 Series (accessed 22 July 2023)
 378 https://www.aphis.usda.gov/animal_health/vet_biologics/publications (accessed 4 July 2022).

379 To test for ability to support growth in the absence of the test material, media should be
 380 inoculated with 10–100 viable control organisms of the suggested ATCC strains listed in Table
 381 2 and incubated according to the conditions specified.

382 To test for ability of the culture media to support growth in the presence of the test material,
 383 containers should be inoculated simultaneously with both the test material and 10–100 viable
 384 control organisms. The number of containers used should be at least one-half the number
 385 used to test the product or product component. The test media are satisfactory if clear
 386 evidence of growth of the control organisms appears in all inoculated media containers within
 387 7 days. In the event that growth is evident, the organism should be identified to confirm that it

¹ American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110-2209, USA.

388 is the organism originally added to the medium. The sterility test is considered invalid if any of
389 the media show inadequate growth response, or if the organism recovered, is not the organism
390 used to inoculate the material.

391 If the material being tested renders the medium turbid so that the presence or absence of
392 microbial growth cannot be readily determined by visual examination, 14 days after the
393 beginning of incubation transfer portions (each not less than 1 ml) of the medium to fresh
394 vessels of the same medium and then incubate the original and transfer vessels for not less
395 than 4 days.

396 **2.2. General procedure for testing live viral vaccines produced in eggs and administered**
397 **through drinking water, spray, or skin scarification for the presence of bacteria and fungi**

398 ~~Each batch of final container biological should have an average contamination of not more than one~~
399 ~~bacterial or fungal colony per dose for veterinary vaccines. From each container sample, each of two~~
400 ~~Petri dishes are inoculated with vaccine equal to ten doses if the vaccine is recommended for poultry,~~
401 ~~or one dose if recommended for other animals. To each plate 20 ml of brain heart infusion agar are~~
402 ~~added containing 0.007 IU (International Units) of penicillinase per ml. One plate should be incubated~~
403 ~~at 30–35°C for 7 days and the other at 20–25°C for 14 days. Colony counts are made at the end of~~
404 ~~each incubation period. An average colony count of all the plates representing a batch should be~~
405 ~~made for each incubation condition. If the average count at either incubation condition exceeds one~~
406 ~~colony per dose in the initial test, one retest to rule out faulty technique may be conducted using~~
407 ~~double the number of unopened final containers. If the average count at either incubation condition~~
408 ~~of the final test for a batch exceeds one colony per dose, the batch of vaccine should be considered~~
409 ~~unsatisfactory.~~

410 **2.32. Example of general procedure for testing seed lots of bacteria and live bacterial**
411 **biologicals for purity**

412 Each seed lot of bacteria or batch of live bacterial biological should be tested for purity by inoculation
413 of SCDM, which is incubated at 20–25°C for 14 days, and FTM, which is incubated at 30–35°C for
414 14 days. Using good practices in sterile technique to avoid laboratory contamination, a sterile pipette
415 or syringe and needle is used to aseptically transfer the quantity of biological directly into the two
416 types of culture medium. The minimum ratio of inoculum to culture medium is 1/15. Both positive and
417 negative controls are set up as well.

418 If the inoculum or growth of the bacterial vaccine renders the medium turbid so that the absence of
419 atypical microbial growth cannot be determined by visual examination, subcultures should be made
420 from all turbid tubes on day 3 through until day 11. Subculturing is done by transferring 0.1–1.0 ml to
421 differential broths and agar and incubating for the balance of the 14-day period. Microscopic
422 examination by Gram stain should also be done.

423 If no atypical growth is found in any of the test vessels when compared with a positive control included
424 in the test, the lot of biological may be considered satisfactory for purity. If atypical growth is found
425 but it can be demonstrated by a negative control that the media or technique were faulty, then the
426 first test ~~may~~ should be repeated. If atypical growth is found but there is no evidence invalidating the
427 test, then a retest ~~may~~ should be conducted. Twice the number of biological containers and test
428 vessels of the first test are used in the retest. If no atypical growth is found in the retest, the biological
429 could be considered to be satisfactory for purity but the results from both the initial and retest should
430 be reported for assessment by the individual countries relevant regulatory agency if the laboratory is
431 sure that the first test result was not due to in-laboratory contamination. If atypical growth is found in
432 any of the retest vessels, the biological is considered to be unsatisfactory for purity. If, however, it
433 can be demonstrated by controls that the media or technique of the retest were faulty, then the retest
434 ~~may~~ should be repeated.

435 **2.43. An Example of a specific test procedure for exclusion of *Brucella sp.* including**
436 ***B. abortus* (where general testing is not sufficient) for detection of *Brucella abortus***

437 It should be confirmed that each batch of culture medium supports the growth of *B. abortus* by
438 inoculating plates and flasks of biphasic medium with a known number of cells (around 100) of the
439 fastidious *B. abortus* biovar 2. If the media supports the growth of this biotype it will support all other
440 biovars.

441 Inoculate 1.0 ml of prepared master or working ~~viral~~ live agent or cell seed material (not containing
442 antibiotics) by inoculating 50 µl of the test product into each of 10 flasks containing biphasic medium.
443 At the same time 10 plates of serum dextrose agar (SDA) are inoculated with 50 µl of inoculum and
444 spread with a sterile bent glass Pasteur pipette or hockey stick. An un-inoculated serum dextrose
445 agar plate and a biphasic flask are also set up at the same time as negative controls.

446 For assessment of inhibitory substances 50 µl of previously prepared master or working viral or cell
447 seed material and 10–100 CFU of *B. abortus* are inoculated on to duplicate SDA plates. Positive
448 controls are prepared by inoculating 10–100 CFU of *B. abortus* on to duplicate SDA plates.

449 All plates and flasks are incubated at 37°C in a 5–10% CO₂ environment. Plates are incubated with
450 the agar uppermost and flasks with the agar slope vertical. Flasks are incubated with the cap loose.

451 Plates are checked for growth of colonies at days 4 and 8 of incubation. The biphasic medium is
452 examined every 4 to 7 days for 28 days. After each examination of the flasks, they are tilted so that
453 the liquid phase runs over the solid phase, then righted and returned to the incubator.

454 During the incubation period, SDA plates with positive control and test material are visually compared
455 with plates with the positive control only and if there is no inhibition of growth of the organism in the
456 presence of the test material, the interference testing test is successful, and testing can be assured
457 to be sensitive.

458 Any signs of growth of suspicious contaminating microorganisms on SDA plates, cloudiness or
459 colonies in biphasic flasks require follow-up testing by PCR to confirm whether *B. abortus* is present.

460 **2.54. An Example of a general procedure for detection of *Salmonella* contamination**

461 Each batch of ~~live virus~~ biological reagents made in eggs should be free from contamination with
462 *Salmonella*. This testing must be done before bacteriostatic or bactericidal agents are added. Five
463 samples of each batch should be tested; 5 ml or one-half of the container contents, whichever is the
464 lesser, of the sample should be used to inoculate 100 ml of tryptose broth and tetrathionate broth.
465 The inoculated broths should be incubated for 18–24 hours at 35–37°C. Transfers from these broths
466 should be made on to MacConkey and *Salmonella–Shigella* agar, incubated for 18–24 hours, and
467 examined. If no growth typical of *Salmonella* is noted, the agar plates should be incubated an
468 additional 18–24 hours and again examined. If colonies typical of *Salmonella* are observed, further
469 subculture on to suitable differential media should be made for positive identification. Sensitive PCR
470 tests are available for the detection of *Salmonella* spp. in cultured material. If *Salmonella* is detected,
471 the batch is determined to be unsatisfactory.

472 **3. Example of detection of *Mycoplasma* contamination**

473 **3.1. An example of a general-specific procedure for ~~detection-exclusion~~ of *Mycoplasma***
474 ***mycoides* subsp. *mycoides* (where general testing is not sufficient)**

475 Each batch of ~~live viral vaccine~~, each lot of master seed virus (MSV), each lot of primary and master
476 cell stock (MCS), and all ingredients of animal origin not steam-sterilised should be tested for the
477 absence of mycoplasmas. Solid and liquid media that will support the growth of small numbers of
478 test organisms, such as typical contaminating organisms *Acholeplasma laidlawii*, *Mycoplasma*
479 *arginini*, *M. fermentans*, *M. hyorhinis*, *M. orale*, and *M. synoviae* should be used. The nutritive
480 properties of the solid medium should be such that no fewer than 100 CFU should occur with each
481 test organism when approximately 100–200 CFUs are inoculated per plate. An appropriate colour

482 change should occur in the liquid media when approximately 20–40 CFUs of each test organism are
483 inoculated. The ability of the culture media to support growth in the presence of product should be
484 validated for each product to be tested, and for each new batch or lot of culture media.

485 One sample of each lot of vaccine, e.g. MSV or MGS, should be tested. Four plates of solid medium
486 are inoculated with 0.25 ml of the sample being tested, and 10 ml of the sample inoculated into 100
487 ml of the liquid medium. An alternative is to inoculate each of the plates with 0.1 ml and to inoculate
488 100 ml of liquid medium with 1 ml of the sample being tested. Two plates are incubated at 35–37°C
489 aerobically (an atmosphere of air containing 5–10% CO₂ and adequate humidity) and two plates are
490 incubated anaerobically (an atmosphere of nitrogen containing 5–10% CO₂ and adequate humidity)
491 for 14 days. On day 3 or day 4 after inoculation, 0.25 ml from the liquid media are subcultured on to
492 two plates of solid media. One plate is incubated aerobically and the second anaerobically at 35–
493 37°C for 14 days. The subculture procedure is repeated on day 6, 7, or 8 and again on day 13 or 14.
494 An alternative method is to subculture on days 3, 5, 10, and 14 on to a plate of solid medium. All the
495 subculture plates are incubated for 10 days except for the 14-day subculture, which is incubated for
496 14 days. Liquid media is observed every 2–3 days and, if any colour change occurs, has to be
497 subcultured immediately.

498 **3.2. Interpretation of *Mycoplasma* test results**

499 At the end of the incubation period (total 28 days), examine all the inoculated solid media
500 microscopically for the presence of mycoplasma colonies. The test sample passes the test if the
501 growth of mycoplasma colonies has occurred on the positive controls, and if growth has not occurred
502 on any of the solid media inoculated with the test material. If at any stage of the test, more than one
503 plate is contaminated with bacteria or fungi, or is broken, the test is invalid and should be repeated.
504 If mycoplasma colonies are found on any agar plate, a suitable confirmatory test on the colonies
505 should be conducted, such as PCR. Some mycoplasmas cannot be cultivated, in which case the
506 MSV and MGS have to be tested using an indicator cell line such as Vero cells, DNA staining, or
507 PCR methods.

508 Further detailed procedures can be found in Veterinary Medicinal Products, VICH GL34:
509 [http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2013/03/WC500140](http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2013/03/WC500140352.pdf)
510 [352.pdf](http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2013/03/WC500140352.pdf)

511 Prior to beginning testing it is necessary to determine that each batch of media promotes the growth
512 of *M. mycoides* subsp. *mycoides* SC (*MmmSC*) type strain PG1. General mycoplasma broth and
513 agar are used but contain porcine serum as a supplement. Each batch of broth and agar is inoculated
514 with 10–100 CFU of *MmmSC*. The solid medium is suitable if adequate growth of *MmmSC* is found
515 after 3–7 days' incubation at 37°C in 5–10% CO₂. The liquid medium is suitable if the growth on the
516 agar plates subcultured from the broth is found by at least the first subculture. If reduced growth
517 occurs another batch of media should be obtained and retested.

518 1 ml of cell or virus seed to be tested is inoculated into 9 ml of the liquid medium and 100 µl on to
519 solid mycoplasma agar. The volume of the product is inoculated so that it is not more than 10% of
520 the volume of the medium. The liquid medium is incubated at 37°C in 5–10% CO₂ and 100 µl of broth
521 is subcultured on to agar at days 7, 14 and 21. The agar plates are incubated at 37°C in 5–10% CO₂
522 for no fewer than 14 days, except those corresponding to day 21 subculture, which are incubated for
523 7 days. An un-inoculated mycoplasma broth and agar plate are incubated as negative controls. For
524 assessment of inhibitory substances, inoculate 1 ml of sample to be tested into 9 ml of the liquid
525 medium and 100 µl on to solid medium and add 10–100 CFU of *MmmSC* to each. Prepare positive
526 control by inoculating 9 ml of mycoplasma broth and a mycoplasma agar plate with 10–100 CFU of
527 *MmmSC*. Incubate as for samples and negative controls.

528 During incubation time, visually compare the broth of the positive control with sample present with
529 the positive control broth and, if there is no inhibition of the organism either the product possesses
530 no antimicrobial activity under the conditions of the test, or such activity has been satisfactorily
531 eliminated by dilution. If no growth or reduced growth of *MmmSC* is seen in the liquid and solid
532 medium with test sample when compared with the positive control, the product possesses

533 antimicrobial activity, and the test is not satisfactory. Modifications of the conditions to eliminate the
534 antimicrobial activity and repeat test are required.

535 If antimicrobial activity is present it is necessary to dilute the test product further. Repeat the test
536 above using 1.0 ml of sample in 39 ml of mycoplasma broth and then inoculate with 10–100 CFU of
537 *MmmSC* and incubate as above. All broths and plates are examined for obvious evidence of growth.
538 Evidence of growth can be determined by comparing the test culture with the negative control, the
539 positive control, and the inhibition control.

540 If evidence of microbial growth is found in the test samples the contaminating bacterium will be
541 identified and confirmed as *MmmSC* by specific PCR assay.

542 **3.2 General testing for exclusion of *Mycoplasma* sp.**

543 General testing for exclusion of *Mycoplasma* sp. that are less fastidious may require up to 28 days
544 in culture, using general mycoplasma media. Some mycoplasmas cannot be cultivated, in which
545 case the live biological sample will have to be tested using an indicator cell line such as Vero cells,
546 DNA staining, or PCR methods.

547 Further detailed procedures can be found in Veterinary Medicinal Products, VICH GL34:
548 [http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2013/03/WC500140](http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2013/03/WC500140352.pdf)
549 [352.pdf](https://www.ema.europa.eu/en/vich-gl34-biologicals-testing-detection-mycoplasma-contamination-scientific-guideline) [https://www.ema.europa.eu/en/vich-gl34-biologicals-testing-detection-mycoplasma-](https://www.ema.europa.eu/en/vich-gl34-biologicals-testing-detection-mycoplasma-contamination-scientific-guideline)
550 [contamination-scientific-guideline](https://www.ema.europa.eu/en/vich-gl34-biologicals-testing-detection-mycoplasma-contamination-scientific-guideline)

551 and

552 USDA SAM 910: https://www.aphis.usda.gov/animal_health/vet_biologics/publications/910.pdf,
553 (both accessed 25 July 2023).

554 **4. Example of detection of rickettsia and protozoa**

555 There are no general test procedures for exclusion of rickettsia or protozoa. Procedures to exclude specific
556 agents of concern such as *Coxiella burnetti* (Q fever), *Ehrlichia canis*, *Trypanosoma evansi* and *Babesia caballi*
557 can be found for example, in the Review of Published Tests to detect pathogens in veterinary vaccines
558 Intended for Importation into Australia ([Australian Government](#) Department of Agriculture ~~[of Australia]~~, Forest
559 and Fisheries (2013)). The review is based on the reading and interpretation of applicable published papers
560 from reputable journals and are regarded as examples of sensitive methods for detection of specified agents.

561 **4.1. An Example of a specific test protocol based on published methods for exclusion of *Babesia*** 562 ***caballi* and *Theileria equi***

563 *Babesia caballi* and *Theileria equi* can be cultured *in vitro* in 10% equine red blood cells (RBC) in
564 supportive medium supplemented with 40% horse serum and in a micro-aerophilic environment.
565 Culture isolation of *T. equi* is more sensitive than for *B. caballi*. Giemsa-stained blood smears are
566 prepared from cultures daily for 7 days (Avarzed *et al.*, 1997; Ikadai *et al.*, 2001). *Babesia caballi* is
567 characterised by paired merozoites connected at one end. *Theileria equi* is characterised by a tetrad
568 formation of merozoites or 'Maltese cross'. Confirmation of the diagnosis is by PCR (see Chapter
569 2.5.8 *Equine piroplasmosis*). Molecular diagnosis is recommended for the testing of biological
570 products that do not contain whole blood or organs. Molecular diagnosis by PCR or loop-mediated
571 isothermal amplification (LAMP) assay are the most sensitive and specific testing methods for
572 detection of the pathogens of equine *piroplasmosis* (Alhassan *et al.*, 2007).

573 **5. Example of detection of virus viruses in biological materials**

574 In brief, general testing usually includes the use of continuous and primary cell lines of the source species,
575 e.g. cells of known susceptibility to the likely viral contaminants, which are inoculated for usually a period of
576 up to 3–4 weeks with weekly subcultures. Virus seeds also require testing on a primary cell line of the species
577 in which the final product is intended. At Day 21 or 28, assessment of the monolayers is done using H&E

578 appropriate histology staining procedures to assess CPE, and haemadsorption with guinea-pig and chicken
579 RBC to assess the presence of haemadsorbing agents. Note that general testing is useful as a screening tool
580 though not sufficiently sensitive enough to detect all viruses of concern to all countries.

581 Specific testing requires test material to be inoculated on to sensitive, susceptible cells lines for the virus to be
582 excluded; the amplification process in cell culture is usually up to 28 days but depending of on the virus, may
583 require longer culturing times. Detection of specific viral contaminants is by recognition of CPE in conjunction
584 with more sensitive antigen detection or molecular tests such as FAT and PCR and ELISA after the
585 amplification process in cell culture is completed.

586 All testing using cell lines to amplify for target viruses is contingent on the sensitivity of the cells for the target
587 agent and the ability to recognise the presence of the agent in the cells. The quality, characteristics, and virus
588 permissibility profile of cell lines in use should be determined as fit for purpose and appropriately maintained.
589 ~~Positive and negative controls should be used at all passages of cell culture to determine sensitivity and~~
590 ~~specificity. Interference testing should be performed at first pass to ensure that the test sample does not inhibit~~
591 ~~the growth of the virus being excluded for.~~

592 **5.1. An example of general testing for the exclusion of viruses from virus and cell seed stocks** 593 **used in production of veterinary vaccines**

594 ~~If the test virus inoculum is cytopathogenic. If a virus seed is known to cause cytopathic effect (CPE) in a~~
595 ~~permissive cell line, the effect must be specifically neutralised without affecting the likelihood of isolation of the~~
596 ~~target agent. For affected cell type, 1 ml of the test master (or working) virus seed (MVS) is thawed or~~
597 ~~reconstituted and neutralised with the addition of 1 ml mono-specific antiserum. The serum must be shown to~~
598 ~~be free from antibodies against any agents for which the test is intended to detect. Antiserum must-should~~
599 ~~be tested for nonspecific inhibiting affects. For a general test, this can be difficult to ascertain. Serum should be~~
600 ~~of sufficiently high titre to neutralise the seed virus effectively with the use of an approximately equal volume~~
601 ~~or less of serum. A microplate block titration is used-useful to determine the titre-amount of the antiserum~~
602 ~~required to neutralise the MVS a known amount of concern. The antiserum CPE causing virus seed. This is~~
603 ~~allowed to neutralise the MVS at 37°C for 1 hour. The MVS and antiserum mixture is then inoculated on to a~~
604 ~~75 cm² flask with appropriate cells. If the MVS is known to be high-titred or difficult to neutralise, the blocking~~
605 ~~antiserum can be added to the growth medium at a final concentration done in the normal conditions required~~
606 ~~of 1–2% each test system (e.g. time, temperature, cell type etc.).~~

607 ~~Master cell-~~If a virus seed is known to be high-titred or difficult to neutralise, antiserum can be added to the
608 growth medium in a test system at a final concentration of 1–2%.

609 Cell seed stocks do not require a neutralisation process.

610 **5.1. Example of general testing procedures for the exclusion of viruses from virus and cell seed** 611 **stocks used in production of veterinary vaccines**

612 **5.1.1 Example of amplification in cell culture**

613 ~~The cells should be passaged weekly up to a 28-day period. Continuous and primary, 75 cm²~~
614 ~~area monolayers of the source species (and intended species as applicable) are infected with~~
615 ~~1 ml of seed stocks and passaged weekly for between up to 21–28-days. Depending on the~~
616 ~~procedure followed, monolayers can be subcultured between passes or freeze/thawed to~~
617 ~~disrupt cells. Negative and positive controls should be also set up at each pass using the same~~
618 ~~cell population. Certain relevant viruses may be selected as indicators for sensitivity and~~
619 ~~interference (positive controls) but these will not provide validation for the broader range of~~
620 ~~agents targeted in general testing. The final culture is examined for cytopathology and~~
621 ~~haemadsorption.~~

622 **5.1.2 Example of general detection procedures: cytopathology**

623 May–Grünwald–Giemsa or H&E staining procedures are used to assess for cytopathological
624 changes associated with virus growth. Monolayers must have a surface area of at least 6 cm²
625 and can be prepared on appropriate chambered tissue culture slides and incubated for 7 days.
626 The plastic wells of the slides are removed leaving the rubber gasket attached to the slide.

627 The slides are rinsed in Dulbecco's phosphate buffered saline (PBS), fixed in acetone,
628 methanol or formalin depending on the stain used and placed on a staining rack. For May-
629 Grünwald-Giemsa staining: the slides are stained for 15 minutes at room temperature with
630 May-Grünwald stain diluted 1/5 with absolute methanol. The May-Grünwald stain is removed
631 by inverting the slides. The slides are then stained for 20 minutes with Giemsa stain diluted
632 1/15 in deionised water. The Giemsa stain is removed by inverting the slides and rinsing them
633 in deionised water for 10-20 seconds. The slides are air-dried and mounted with a coverslip
634 using paraffin oil. The May-Grünwald-Giemsa stain differentially stains ribonucleoprotein
635 (RNP); DNA RNP stains red-purple, while RNA RNP stains blue. The monolayers are
636 examined with a conventional microscope for the presence of inclusion bodies, an abnormal
637 number of giant cells, or other cytopathology attributable to a viral contaminant of the test
638 product. The inoculated monolayers are compared with suitable control non-inoculated
639 monolayers. If specific cytopathology attributable to an extraneous virus is found, results are
640 reported, and additional specific testing may be conducted.

641 **5.1.3 Example of general detection procedures: haemadsorption**

642 Testing for haemadsorption ~~uses~~ requires the use of 75 cm² area monolayers established in
643 tissue culture flasks after the 28-day passage period described above. Guinea-pig, chicken,
644 and any other blood for use in this assay is collected in an equal volume of Alsever's solution
645 and may be stored at 4°C for up to 7 days. Immediately prior to use, the stored erythrocytes
646 are again washed by adding 5 ml of blood in Alsever's solution to 45 ml of calcium and
647 magnesium-free PBS (PBSA) and centrifuging in a 50 ml centrifuge tube at 500 **g** for 10
648 minutes. The supernatant is aspirated, and the erythrocytes are suspended in PBSA and re-
649 centrifuged. This washing procedure is repeated at least twice until the supernatant is clear.
650 Erythrocytes from each species are combined by adding 0.1 ml of each type of packed blood
651 cells to 100 ml of PBSA. The erythrocytes from different species may be kept separate or
652 combined, as desired. To each flask, add 5 ml of the erythrocyte suspension, and incubate
653 the flasks at 4°C for 30 minutes. Monolayers are washed twice with PBSA and examined for
654 haemadsorption. If no haemadsorption is apparent, 5 ml of ~~the fresh~~ erythrocyte suspension
655 is added to each flask; the flasks are incubated at 20-25°C (room temperature) for 30 minutes,
656 rinsed as before, and examined for haemadsorption. Separate flasks may be used for each
657 incubation temperature if desired. Monolayers are examined for the presence of
658 haemadsorption using an illuminated light box and microscopically. Non-inoculated
659 monolayers are used as negative controls. The PBSA and fresh erythrocytes should prevent
660 most nonspecific haemadsorption from occurring. If specific haemadsorption attributable to an
661 extraneous agent is found, results are reported, and additional specific testing may be
662 conducted.

663 ~~Specific testing requires specialised test procedures that are sensitive to amplifying a particular agent~~
664 ~~in culture and then detection of that agent by means of fluorescence, antigen capture ELISA or PCR;~~
665 ~~whichever is more sensitive. Specific testing is usually required when general procedures are not~~
666 ~~adequate for effective exclusion of more fastidious, viruses. Some examples are listed in Table 1.~~

667 **5.2. An Examples of specific virus agent exclusion testing from of biologicals used in the** 668 **production of veterinary vaccines**

669 **5.2.1. Example of porcine epidemic diarrhoea virus (PEDV)**

670 Trypsin presence is required at inoculation and in the culture medium for isolation of porcine
671 epidemic diarrhoea virus (PEDV) in Vero cells (CCL81, ATCC) to ensure the virus can enter
672 host cells. Just confluent monolayers (100%) are required; as under confluent monolayers
673 (<90%) are more sensitive to the presence of trypsin ~~and will be destroyed well before the 7~~
674 ~~days required for each passage in culture~~. An over confluent or aging monolayer will not be
675 sensitive for growth of PEDV. Maintenance media (MM) formulation consists of Earle's MEM
676 (minimal essential medium) (with 5.6 M HEPES [N-2-hydroxyethylpiperazine, N-2-
677 ethanesulphonic acid] and glutamine) + 0.3% Tryptose phosphate broth, 0.02% yeast extract
678 and 4 µg/ml TPCK treated trypsin. The addition of the trypsin into ~~the~~ MM should occur on the
679 day the media is to be used.

680 Prior to inoculation, confluent 75 cm² monolayers are washed twice with the MM (with trypsin
681 added) to remove growth media containing FCS. Virus or cell seed (1 ml) is added with 1 ml
682 of MM to each monolayer; incubate at 37°C for 2 hours, then add 30 ml/flask of MM. Negative
683 control monolayers of the same size are set up prior to inoculation of test material. Positive
684 and interference controls are set up last, and where possible, in a separate laboratory area to
685 avoid contamination. Assessment for sensitivity and interfering substances requires
686 assessment use of PEDV reference virus of known titre. A control for interference using co-
687 inoculation of test sample and PEDV needs only to be set up on the first pass. Positive controls
688 must should be set up at every pass to ensure each monolayer used gives expected sensitivity.
689 PEDV virus is titrated in log dilutions starting at 10⁻¹ to 10⁻⁶ in MM (depending ~~of~~ on the
690 endpoint titre of reference virus) in duplicate rows of 6 wells of a 24-well tissue culture plate.
691 For the interference test, PEDV is titrated in the same dilution series but using MM spiked with
692 a 10% volume of test material. Decant off the growth media and discard. Wash plates to ensure
693 no FCS is present. Two washes using approximately 400 µl/well MM (with trypsin added) are
694 sufficient.

695 Add 100 µl of diluted virus on to each of two duplicate wells. Rock inoculated plates to distribute
696 the inoculum evenly over the surface of the monolayer. Incubate at 37°C with 5% CO₂ for
697 2 hours then add a further 1 ml volumes/well of MM.

698 After 7 days, 75 cm² monolayers have cells disrupted using two freeze–thaw cycles at –80°C.
699 Positive control plates are read for end-point titres, and these are compared with virus in the
700 presence of test material to ensure titres are comparable and interference has not occurred.
701 Freeze–thaw lysates are clarified at 2000 **g** for 5 minutes and re-passed on to newly formed
702 monolayers as for the first passage. Passages are repeated until a total of four passages are
703 completed at which point cell lysates are assessed by PCR for detection of PEDV and day 7
704 monolayers in 24-well plates are fixed and stained ~~by IFA for FAT~~. If a seed virus is to be
705 tested and requires neutralisation using antiserum, extra care in the isolation of PEDV needs
706 to be considered. Trypsin is rendered inactive in the presence of serum proteins and without
707 trypsin present, PEDV is unable to grow in cell culture grows poorly, or not at all. Washing off
708 the inoculum with two MM washes is required after an extended adsorption time of up to 4
709 hours to ensure acceptable sensitivity.

710 **J.H. INFORMATION TO BE SUBMITTED WHEN**

711 **APPLYING FOR AN IMPORT LICENCE**

712 When undertaking risk analysis for biologicals, Veterinary Authorities should follow the *Terrestrial Code*
713 *Manual*, and the manufacturer should follow the requirements of the importing country. Requirements for each
714 importing country should be accessible and published online. The manufacturer or the Veterinary Authority of
715 the exporting country should make available detailed information, in confidence if as necessary, on the source
716 of the materials used in the manufacture of the product (e.g. substrates). They should make available details
717 of the method of manufacture (and where appropriate inactivation) of the substrates and component materials,
718 the quality assurance procedures for each step in the process, final product testing regimes, and the
719 pharmacopoeia with which the product must conform in the country of origin. They should also make available
720 challenge organisms, their biotypes and reference sera, and other means of appropriate product testing.

721 For detailed examples of a risk-based assessment of veterinary biologicals for import into a country refer to:

- 722 • European Commission (2015). The Rules Governing Medicinal Products in the European Union.
723 Eudralex. Volume 6. Notice to applicants and regulatory guidelines for medicinal products for veterinary
724 use.
- 725 • Department of Agriculture, Forest and Fisheries of Australia (2021b). Live veterinary vaccines Summary
726 of information required for biosecurity risk assessment, Version 6 and Inactivated veterinary vaccines,
727 Version 8.

728 • Outline of the Regulatory System of Veterinary Drugs in Japan (2015) Assurance of the Quality, Efficacy,
729 and Safety Based on the Law for Ensuring the Quality, Efficacy, and Safety of Drugs and Medical Devices.

730 • Ministry of Agriculture and Rural Affairs, China (People's Rep. of), Regulations on the Administration of
731 Veterinary drugs (revised in 2020).

732 When applying for an import licence other regulatory requirements may need to be addressed depending on
733 the type of sample and if the sample needs to be shipped out of country to a testing laboratory. For example,
734 cell seeds may come under certain requirements for permits such as the Convention for International Trade in
735 Endangered Species of Wild Fauna and Flora (CITES), where a cell line is derived from an endangered
736 species, e.g. the cell line and its derivatives. Applying for such a permit is time consuming and requires input
737 from both the exporting and importing country.

738 Genetically modified organisms (GMOs) are becoming more frequent in use with changes in manufacturing
739 technologies and specialised, time-consuming procedures need to be in place. A laboratory that accepts a
740 GMO product for testing shall follow the procedures of the Office of the Gene Regulator (OGTR) to allow the
741 GMO to be dealt with.

742 **I. RISK ANALYSIS PROCESS**

743 Risk analysis should be as objective and transparent as possible and should be performed in accordance with
744 Section 2 of the *Terrestrial Code*, and certification in line with Section 5 of the *Terrestrial Code*. Of necessity,
745 assessment of the country and commodity factors and risk reduction measures will be based largely on
746 manufacturers' data. These data depend on quality assurance at all stages of manufacture, rather than on
747 testing of the final product alone.

748 Domestic exposure may be influenced by the approved usage of the product. Veterinary Authorities may place
749 limits on usage of some products (e.g. restricting usage to institutions of appropriate biosecurity).

750 **I.J. BIOCONTAINMENT**

751 Suitable biocontainment may be necessary for many forms of biologicals. In particular, the importation of exotic
752 micro-organisms should be carried out in accordance with Chapter 1.1.4 *Biosafety and biosecurity: standard*
753 *for managing biological risk in the veterinary laboratory and animal facilities*.

754 Laboratories using high risk agents should have well researched and documented risk assessments in place
755 prior to working with such agents to ensure the safety of their staff and laboratory.

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FURTHER READING

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NB: FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2017.

Annexe 6. Chapter 2.2.4. 'Measurement uncertainty'

CHAPTER 2.2.4.

MEASUREMENT UNCERTAINTY

INTRODUCTION

The WOAH Validation Recommendations provide detailed information and examples in support of the ~~WOAH Validation Standard that is published as Chapter 1.1.6 Principles and methods of Validation of diagnostic assays for infectious diseases of terrestrial animals this Terrestrial Manual, or Chapter 1.1.2 of the Aquatic Manual. The Term "WOAH Validation Standard" in this chapter should be taken as referring to those chapters.~~

Estimation of measurement uncertainty (MU), ~~sometimes termed measurement imprecision~~, is a requirement for testing laboratories based on international quality standards such as ISO/IEC 17025-2005, ~~2017~~ General requirements for the competence of testing and calibration laboratories (ISO/IEC 17025). The measurement process for detection of an analyte in a diagnostic sample is not entirely reproducible and hence there is no exact value that can be associated with the measured analyte. Therefore, the result is most accurately expressed as an estimate ~~together with an associated level of imprecision level~~. This imprecision is the measurement uncertainty (MU). MU is limited to the measurement process of quantitative tests. The approach described here is known as "top-down" or "control sample" because it uses a weak positive control sample and expresses the MU result at the cut-off diagnostic threshold, where it most matters. It is not a question of whether the measurement is appropriate and fit for whatever use to which it may be applied. It is not an alternative to test validation but is rightly considered a component of that process (see ~~the WOAH Validation Standard, chapter 1.1.6 Section B.1.1 Repeatability~~).

A. THE NECESSITY OF DETERMINING MU

To assure compliance with ISO/IEC 17025-2005-~~2017~~ requirements, national accreditation bodies for diagnostic testing laboratories require laboratories to calculate MU estimates for accredited test methods that produce quantitative results, e.g. optical densities (OD), percentage of positivity or inhibition (PP, PI), titres, cycle threshold (CT) values, etc. This includes tests where numeric results are calculated and then ~~are~~ expressed as a positive or negative result at a cut-off value. For the purpose of estimating MU in serology and reverse transcriptase polymerase chain reaction (RT-PCR), suitable statistical measures are mean target values ± 2 standard deviations (SD), which is an approximately equal to a 95% confidence reference interval (C-RI), relative standard deviation (RSD = SD / mean of replicates) and coefficient of variation (CV = RSD \times 100%). Examples provided below assume normal distribution of data. Alternative methods are available that are less sensitive to both that assumption and to the presence of outliers; they are not illustrated here The concept of MU does not apply to strictly binary (qualitative) results (positive or negative).

35 1. Samples for use in determining MU

36 Repeatability is the level of agreement between results of replicates of a sample both within and between runs
37 of the same test method in a given laboratory. During assay development, repeatability is estimated by
38 evaluating variation in results of independent replicates from a minimum of three (preferably five) samples
39 representing analyte activity within the operating range of the assay (see ~~the WOAHS Validation Standard,~~
40 Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*, Sections A.2.5
41 *Robustness* and B.1.1 *Repeatability*, and Chapter 2.2.6 *Selection and use of reference samples and panels*,
42 Section 3.4 *A.4.2*). Typically, the variation in replicate results is expressed as RSD or CV. The significant
43 feature is that repeatability studies can be used to define the expected precision of the assay in the detection
44 of a range of analyte concentrations.

45 The use of internal quality or process controls over a range of expected results has become part of daily quality
46 control and quality assurance operations of accredited facilities (see ~~the WOAHS Validation Standard, chapter~~
47 1.1.6, Sections A.2.6 *Calibration of the assay to standard reagents* and B.5.1 *Monitoring the assay*, and
48 Chapter 2.2.6, Section 4.4 *C.1*). These results provide a continuous monitor relative to different aspects of
49 repeatability, e.g. intra- and inter-assay variation, intra- and inter-operator variation and intra- and inter-batch
50 variation, which, when subjected to statistical analysis, provide an expression of the level of robustness
51 (precision) of a test procedure. The monitoring of assay quality control parameters for repeatability provides
52 evidence that the assay is or is not performing as expected. For control samples to provide valid inferences
53 about assay precision, they should be treated in exactly the same way as test samples in each run of the
54 assay, e.g. including sample preparation such as extraction steps or dilution of serum samples for an antibody
55 enzyme-linked immunosorbent assay (ELISA).

56 The variation of the results for control samples can also be used as an estimate of those combined sources of
57 uncertainty and is called the “top-down” approach. This approach recognises that the components of precision
58 will be manifest in the ultimate measurement. So monitoring the precision of the measurement over time will
59 effectively show the combined effects of the imprecision associated with component steps.

60 The imprecision or uncertainty of the measurement process associated with a test result becomes increasingly
61 more important the closer the test value is to the diagnostic cut-off value. This is because an interpretation is
62 made relative to the assay threshold regarding the status of the test result as positive, negative, or inconclusive
63 (as will be described in the following example). In this context, low-weak positive samples, like those used in
64 repeatability studies or as the low-weak positive control, are most appropriate for estimation of MU. The
65 rationale being that MU, which is a function of assay precision, is most critical at decision-making points (i.e.
66 thresholds or cut-offs), which are usually near the lower limit of detection for the assay. In this chapter, the
67 application of MU with respect to cut-off (threshold) values, whether recommended by test-kit manufacturers
68 or determined in the diagnostic laboratory, is described.

69 MU, using the top-down approach, ideally requires long-term accumulated data from a weak positive control
70 sample after multiple test runs over time, with multiple operators and variable conditions. The examples given
71 below are based on 10 data points but higher numbers will increase robustness.

72 2. Example of MU calculations in ELISA serology

73 For most antibody detection tests, it is important to remember that the majority of tests are measurements of
74 antibody activity relative to a threshold against which a dichotomous interpretation of positive or negative is
75 applied. This is important because it helps to decide where application of MU is appropriate. In serology,
76 uncertainty is frequently most relevant at the threshold between positive and negative determinations. Results
77 falling into this zone are also described as intermediate, inconclusive, suspicious or equivocal (see ~~the WOAHS~~
78 *Validation Standard, chapter 1.1.6, Section B.2.4 *Selection of a cut-off (threshold) value for classification of**
79 *test results*).

80 A limited data set from a competitive ELISA for antibody to avian influenza virus is used as an example of a
81 “top-down” approach for serology. A low-weak positive control sample was used to calculate MU at the cut-off
82 level¹.

83 2.1. Method of expression of MU

84 As the uncertainty is to be estimated at the threshold, which is not necessarily the reaction level of
85 the low-weak positive control serum, the relative standard deviation (RSD), or coefficient of variation
86 (CV), if expressed as a percentage, provides a convenient transformation:

$$\text{RSD (X)} = \text{SD (X)} / \text{mean} (\bar{X})$$

87 X represents the set of replicates

88 To simplify assessment, the a suitably transformed result (such as sample-to-positive ratio, per cent
89 inhibition, or background-corrected optical density) is regarded as the assay output result, which is
90 then averaged across the number of replicates (\bar{X}). In the case of this example, a competitive ELISA,
91 results are “normalised” (as defined in the WOAHS Validation Standard, chapter 1.1.6, Section A.2.7
92 ‘Normalising’ test results to a working standard) to a working standard by forming a ratio of all optical
93 density (OD) values to the OD result of a non-reactive (negative) control (OD_N). This ratio is
94 subtracted from 1 to set the level of antibody activity on a positive correlation scale; the greater the
95 level, the greater the calculated value. This adjusted value is expressed as a per cent and referred
96 to as the percentage inhibition or PI value. So for the low-weak positive control serum (OD_{LW}), the
97 transformation to obtain the per cent inhibition values for the low-weak positive control (PI_{LW}) is:

$$\text{PI}_{\text{LW}} = 100 \times [1 - \{\text{OD}_{\text{LW}} / \text{OD}_{\text{N}}\}]$$

98 The relative standard deviation becomes:

$$\text{RSD (PI}_{\text{LW}}) = \text{SD (PI}_{\text{LW}}) / \text{mean (PI}_{\text{LW}})$$

101 2.2. Example

102 A limited data set for the AI competitive ELISA example is shown below. In the experiment, the
103 operator tested the low-weak positive control serum ten times in the same run. Ideally in the
104 application of this “top down” method, a larger data set would be used, which would enable
105 accounting for effects on precision resulting from changes in operator and assay components (other
106 than only the control serum).

107 **Table 1. Top-down or control sample approach for an influenza antibody C-ELISA**

Test	PI (%)
1	56
2	56
3	61
4	64
5	51
6	49
7	59
8	70
9	55
10	42

108 Mean PI = 56.3; Std Dev (SD) = 7.9; Assays (n) = 10

¹ The Australian Government, Department of Agriculture, Fisheries and Forestry, has compiled worked examples for a number of diagnostic tests Available online at: <https://www.agriculture.gov.au/agriculture-land/animal/health/laboratories/tests/measurement-uncertainty> (accessed 22 June 2023)

2.3. Calculating uncertainty

From the limited data set,

$$\text{RSD (PI}_{\text{LW}}) = \text{SD}/\text{Mean} = 7.9/56.3 = 0.14 \text{ (or as coefficient of variation = 14\%)}$$

Expanded uncertainty (U) is the statistic defining the interval within which the value of the measure and is believed to lie within a specified level of confidence, usually 95%. Expanding the uncertainty is done by multiplying the RSD (PI_{LW}) by a factor of 2; this allows the calculation of an approximate 95% confidence reference interval around the threshold value (in this case at PI = 50%), assuming normally distributed data. If data are not normally distributed they must be transformed to fit a normal distribution using a log scale.

$$U \text{ (95\% C-R)} = 2 \times \text{RSD} = 0.28$$

This estimate can then be applied at the threshold level

$$95\% \text{ C-R} = 50 \pm (50 \times 0.28) = 50 \pm 14\%$$

2.4. Interpretation of the results

Any positive result (PI > 50%) that is less than 64% is not positive with 95% confidence. Similarly, a negative result (PI < 50%) that is higher or equal to a PI of 36 is not negative at the 95% confidence level. A sample with a PI between 36% and 64% is within the MU surrounding the threshold value, and thus its diagnostic status is less certain than those of samples with results further from that threshold. This zone of lower confidence may correlate with the “grey zone” or “inconclusive/suspect zone” for interpretation that should be established for all tests (Greiner *et al.*, 1995).

3. Example of MU calculation in molecular tests

3.1. Example

For real-time PCRs, replicates of positive controls with their respective cycle threshold (Ct) values can be used to estimate MU using the top-down approach (Newberry & Colling, 2021). The method of expression follows the same formula as for the ELISA example above. This example uses data from replicate runs of a weak positive control sample (10 runs) of an equine influenza hydrolysis probe assay.

Table 2. Top-down or control sample approach for an equine influenza TaqMan A assay

<u>Test</u>	<u>Ct value</u>
<u>1</u>	<u>33.60</u>
<u>2</u>	<u>33.20</u>
<u>3</u>	<u>33.96</u>
<u>4</u>	<u>33.18</u>
<u>5</u>	<u>33.96</u>
<u>6</u>	<u>32.72</u>
<u>7</u>	<u>33.57</u>
<u>8</u>	<u>33.45</u>
<u>9</u>	<u>32.80</u>
<u>10</u>	<u>33.20</u>

Mean = 33.36; Std Dev (SD) = 0.43; Assay n=10

3.2. Calculating uncertainty

From the limited data set,

$$\text{RSD (PI}_{LW}) = \text{SD}/\text{Mean } 0.43/33.36 = 0.0128 \text{ (or as coefficient of variation = 1.28\%)}$$

Expanded uncertainty (U) is the statistic defining the interval within which the value of the measure and is believed to lie within a specified level of confidence, usually 95%. Expanding the uncertainty is done by multiplying the RSD (PI_{LW}) by a factor of 2; this allows the calculation of an approximate 95% confidence interval around the threshold value (in this case at Ct value = 37), assuming normally distributed data.

$$U \text{ (95\% C-R)} = 2 \times \text{RSD} = 0.0255$$

This estimate can then be applied at the threshold level

$$95\% \text{ C-R} = 37 \pm (37 \times 0.0255) = 37 \pm 0.94$$

The mean cycle threshold (Ct) value after 10 runs is 33.36 and the standard deviation is 0.43. The relative standard deviation is 0.0128. The expanded uncertainty (95% C-R) is 2 × the relative standard deviation = 0.0255. Measurement of uncertainty (MU) is most relevant at the cut-off (Ct = 37) and can be applied by multiplication (37 × 0.0255 = 0.94). Subtraction from the threshold (37-0.94) provides the lower 95% confidence-reference limit (Ct = 36.06) and addition (37+0.94) the upper 95% confidence-reference limit (Ct = 37.94).

3.3. Interpretation of the results

Any positive result (Ct < 37) that is higher than 36 Ct is not positive with 95% confidence. Similarly, any negative result (Ct > 37) that is less than 38 Ct is not negative with 95% confidence. A sample with a Ct between 36 and 38 is within the MU surrounding the threshold value, and thus its diagnostic status is less certain than those of samples with results further from that threshold.

B. OTHER APPLICATIONS

The top-down approach should be broadly applicable for to a range of diagnostic tests including molecular tests. For the calculation of tests using a typical two-fold dilution series for the positive control such as virus neutralisation, complement fixation and haemagglutination inhibition tests geometric mean titre (i.e. geom and expanded [SD] of log base 2 titre values) of the positive control serum should be calculated. Relative standard deviations based on these log scale values may then be applied at the threshold (log base 2) titre, and finally transformed (by antilog) to represent the uncertainty at the threshold. However, in all cases, the approach assumes that the variance about the positive control used to estimate the RSD is proportionally similar at the point of application of the MU, for example at the threshold. If the RSD varies significantly over the measurement scale, the positive control serum used to estimate the MU at the threshold should be selected for an activity level close to that threshold. The Australian Government, Department of Agriculture, Fisheries and Water Resources Forestry, has compiled worked examples for a number of diagnostic tests (see footnote 1). (DAFF, 2010), which are available online at:

<http://www.agriculture.gov.au/animal/health/laboratories/tests/worked-example-measurement>

For quantitative real time PCRs (qPCR) replicates of positive controls with their respective cycle threshold (CT) values can be used to estimate MU using the top-down approach.

Other approaches and variations have been described, i.e. for serological tests (Dimech *et al.*, 2006; Goris *et al.*, 2009; Toussaint *et al.*, 2007). Additional work and policy Central documents are available from the National Pathology Accreditation Advisory Group and Life Science. The central document to MU is are the Guide to the expression of uncertainty in measurement (GUM), ISO/IEC Guide, (1995) and Eurachem/CITAC Guide, 2012 CG 4: Quantifying uncertainty in analytical measurement.

180 **Scope and limitations of the top-down approach**

181 Methods for quantifying uncertainty (addressing MU) for tests vary. When estimating MU for quantitative,
182 biologically based diagnostic tests, where variations in the substrate or matrix have large and unpredictable
183 effects, a top-down approach is recommended (Dimech *et al.*, 2006; Eurachem 2012; Goris *et al.*, 2009;
184 ISO/IEC Guide 98-3:2008; Newberry & Colling, 2021; Standards Council of Canada, 2021; and footnote 1).
185 The advantage of this method is that quality control data are generated during normal test runs and can be
186 used to estimate the precision of the assay and express it at the cut-off. The application at the cut-off depends
187 on the performance of the test at different analyte concentrations, e.g. variation is likely to increase at higher
188 diluted samples. The top-down approach does not identify individual contributors to measurement uncertainty
189 but rather provides an overall estimate. Measurement uncertainty does not replace test validation; however,
190 the validation process includes assessments of repeatability through quality control samples which facilitate
191 calculation of MU.

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230 **NB:** There is a WOAHA Collaborating Centre for
231 Diagnostic Test Validation Science in the Asia-Pacific Region (please consult the WOAHA Web site:
232 <https://www.woah.org/en/what-we-offer/expertise-network/collaborating-centres/#ui-id-3>).
233 Please contact the WOAHA Collaborating Centre for any further information on validation.

234

NB: FIRST ADOPTED IN 2014.

Annexe 7. Chapter 2.2.6. 'Selection and use of reference samples and panels'

CHAPTER 2.2.6.

SELECTION AND USE OF REFERENCE SAMPLES AND PANELS

INTRODUCTION

The WOAH Validation Recommendations provide detailed information and examples in support of ~~the WOAH Validation Standard that is published as Chapter 1.1.6 Principles and methods of Validation of diagnostic assays for infectious diseases of terrestrial animals this Terrestrial Manual, or Chapter 1.1.2 of the Aquatic Manual. The Term "WOAH Validation Standard" in this chapter should be taken as referring to those chapters.~~

Reference samples and panels are essential from the initial proof of concept in the development laboratory through to the maintenance and monitoring of assay performance in the diagnostic laboratory and all of the stages in between. The critical importance of reference samples and panels cannot be over-emphasised. The wrong choice of reference materials can lead to bias and flawed conclusions right from development through to validation and use. Therefore, care must be exercised in selecting reference samples and designing panels.

Fig. 1. Reference samples and panels grouped based on similar characteristics and composition. The topics and alphanumeric subheadings (e.g. Proof of concept, A.2.1) refer to the relevant section in ~~the WOAH Validation Standard, Chapter 1.1.6 Validation of diagnostic assays for infectious diseases of terrestrial animals.~~

Group A Proof of concept, A.2.1. Operating range, A.2.2-3. <u>ASe, B.1.3.</u> Optimisation, A.2.-3-2. Robustness, A.2.5. Preliminary repeatability, A.2.8. Calibration and process control, A.2.6. Process control, A.2.6. ASe, B.1.3. Technical modifications, B.5.2.1. Reagent replacement, B.5.2.3.		Group B Asp, B.1.2. Analytical accuracy, <u>ancillary tests</u> B.1.4. Reference samples and panels Group C Repeatability B.1.1. Preliminary reproducibility, B.2.6 <u>I.</u> Reproducibility, B.3. Proficiency testing, B.5.1.		Group D Standard method comparison, B.2.6. Provisional recognition, B.2.6-7. Biological modifications, B.5.2.2. Group E DSp and DSe Gold standard, B.2.1. Group F DSp and DSe no gold standard B.2.2.
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A_{Sp} = Analytical specificity; A_{Se} = Analytical sensitivity; D_{Sp} = diagnostic specificity; D_{Se} = diagnostic sensitivity

~~As can be seen in Figure 1, Reference samples and/or panels are mentioned throughout the WOAH Validation Standard, chapter 1.1.6. As defined in the glossary of the OIE Quality Standard and Guidelines for Veterinary Laboratories: Infectious Diseases, 'Reference materials are~~

24 *“substances whose properties are sufficiently homogenous and well established to be used for*
25 *the calibration of an apparatus, the assessment of a measurement method, or for assigning*
26 *values to materials”¹. In the context of test method validation, reference materials or samples*
27 *contain the analyte of interest in varying concentrations or ~~activities-reactivities~~ and are used in*
28 *developing and evaluating the candidate assay’s analytical and diagnostic performance*
29 *characteristics. ~~In our case,~~ Analyte means the specific component of a test sample that is*
30 *detected or measured by the test method, e.g. antibody, antigen or nucleic acid. ~~These~~ Reference*
31 *samples may be sera, fluids, tissues, excreta, feed ~~and/or~~ environmental samples that contain*
32 *the analyte of interest and are usually harvested from infected animals and their environments.*
33 *However, in some cases, they may be prepared in the laboratory from an original starting material*
34 *(e.g. a dilution of a high positive serum in negative serum) or perhaps created by spiking the*
35 *chosen matrix with a derived analyte (e.g. a bacterial or viral culture, a recombinant/expressed*
36 *protein, or a genomic construct). Whether natural or prepared, they are used in experiments*
37 *throughout the development process, carry over into the validation pathway and can be used to*
38 *monitor performance throughout the lifespan of the assay.*

39 *~~In Figure 1, reference samples and panels are grouped based on similar characteristics and~~*
40 *~~composition and these groupings will be the basis for the following descriptions. As a cross-~~*
41 *~~reference, the appropriate Section of the OIE Validation Standard is indicated under each~~*
42 *~~particular application of the reference sample or panel.~~*

43 *Reference samples may be used for multiple purposes from the initial stages of development and*
44 *optimisation, through Stage 1 and into continual monitoring and maintenance of the assay.*
45 *Wherever possible, large quantities of ~~these~~ reference samples should be collected or prepared*
46 *and preserved for long-term use. Switching reference samples during the validation process*
47 *introduces an intractable variable that can severely undermine interpretation of experimental data*
48 *and ~~therefore,~~ the integrity of the development and validation process. For assays that may target*
49 *multiple species, the samples should be representative of the primary species of interest. It is*
50 *critical that these samples reflect both the target analyte and the matrix in which it is found in the*
51 *population for which the assay is intended. The reference materials should appropriately*
52 *represent the range of analyte concentration to be detected by the assay.*

53 *~~It is important to emphasise that, no matter~~ Whether reference samples are selected from natural*
54 *sources or prepared in the laboratory, all selection criteria ~~or~~ and preparation procedures, as well*
55 *as testing requirements, need to be fully described and put into document control. Not only is this*
56 *good quality management practice, but it will provide both an enhanced level of continuity and*
57 *confidence throughout the lifespan of the assay. Summaries of the data to be collected and*
58 *documented for reference material can be found in Figure 2. For more detail on best practice and*
59 *quality standards for the documentation of provenance of reference material refer to Watson et*
60 *al. (2021).*

¹ https://www.techlab.fr/Commun/UK_Def_MRC.asp

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Fig. 2. Documentation of reference material should be thorough to ensure i) transparency of intended purpose during assay development; ii) the correct sample types are used in all stages of assay development and validation; iii) accurate replacement of depleted reagents; and iv) appropriate choice of reference material during assay modification and re-validation. Minimum descriptive metadata are listed for pathogen, animal host, tissue type and phase of infection.

<u>Pathogen data</u>	<u>Animal host and sample type data</u>	<u>Phase of Infection data</u>
<ul style="list-style-type: none"> • <u>Strain/isolate</u> • <u>Serotype</u> • <u>Genotype</u> • <u>Lineage</u> • <u>Tests used for characterisation</u> 	<ul style="list-style-type: none"> • <u>Natural infection</u> • <u>Experimental infection and protocol used</u> • <u>Species</u> • <u>Breed</u> • <u>Age</u> • <u>Sex</u> • <u>Reproductive status</u> • <u>Vaccination history</u> • <u>Herd history</u> 	<ul style="list-style-type: none"> • <u>Clinical signs</u> • <u>infection/disease outcome</u> • <u>Antibody profiles</u> • <u>Pathogen loading and shedding</u> • <u>Tests used to determine status of disease/infection (case definition)</u> • <u>Time post-experimental infection</u>
	<ul style="list-style-type: none"> • <u>Tissue type/s (matrix) used</u> • <u>For spiked samples – detail source of analyte and diluent (matrix) used</u> • <u>Details relating to pooling of samples</u> 	

67

A. GROUP A

68 The question of pooling of samples to create a reference sample is often asked. If reference material is
69 harvested from a single animal, it is important to ascertain whether or not it is representative of a typical course
70 and stage of infection within the context of the population to be tested. If not, this could lead to bias and flawed
71 conclusions related to validation. Pooling is a good alternative but it is imperative to pool from animals that are
72 in a similar phase of infection. This is particularly important for antibody detection systems. Pooling also
73 addresses the issue of the larger quantities of reference material to be stored for long term use, especially
74 when dealing with smaller host species. Before pooling any samples, it is preferable that they be independently
75 tested to demonstrate that they are similar with respect to analyte concentration and/or reactivity. There should
76 be an assessment following pooling to ensure that unforeseen interference is not introduced by the pooling of
77 multiple samples, for example differing blood types or antibody composition within the independent samples
78 could cross-react within the pool, thus causing the pooled sample to behave differently in the specified assay
79 than the individual samples when tested independently.

80 It is often difficult to obtain individual samples that truly represent analyte concentrations or reactivities across
81 the spectrum of the expected range. Given the dynamics of many infections or responses to pathogens,
82 intermediate ranges are often very transient. In the case of antibody responses, early infection phases in
83 individual animals often result in highly variable and heterogeneous populations of antibody isotypes and
84 avidities. In general, these do not make good reference samples for assessing the analytical characteristics of
85 an assay. They are nonetheless important for different types of reference panels as will be discussed later.
86 For most applications in Group A, it is acceptable to use prepared samples that are spiked with known
87 concentrations of analyte or a dilution series of a high positive in negative matrix to create a range of
88 concentrations.

89 Whether natural or prepared, reference samples should represent the anticipated range of analyte
90 concentrations, from low-weak to high-strong positive, which would be expected during a typical course of
91 infection. A negative reference sample should be included as a background monitor. If a negative (matrix) is
92 used as diluent for preparation of a positive reference sample (e.g. a negative serum used to dilute a high

93 positive serum or tissue spiked with a construct), that negative should definitely be included as the negative
94 reference sample.

95 ~~As mentioned above, all reference samples should be well characterised. This includes documentation on both
96 the pathogen and donor host. For pathogens, this may include details related to strain, serotype, genotype,
97 lineage, etc. The source of the host material should be well described with respect to species, breed, age, sex,
98 reproductive status, vaccination history, herd history, etc. Wherever possible, the phase of infection should be
99 noted. This could include details related to clinical signs, antibody profiles, pathogen load or shedding, etc.
100 Equally important, tests that are used to determine disease/infection status need to be well documented (see
101 Section E of this chapter for further explanation). In some cases, experimental infection/exposure may be the
102 only viable option for the production of reference material. In this case, all of the above considerations plus the
103 experimental protocol should be detailed.~~

104 Above all else, natural or prepared, reference materials must be unequivocal with respect to their status as
105 representing either a true positive or a true negative sample. This may require that the status be confirmed
106 using another test or battery of tests. For example, many antibody reference sera are characterised using
107 multiple serological tests. This provides not only confidence but additional documented characteristics that
108 may be required when attempting to replace or duplicate this reference material in the future.

109 Recommendations regarding stability and storage of reference materials are available:
110 <https://www.woah.org/en/what-we-offer/veterinary-products/#ui-id-4>

111 **1. Proof of concept (WOAH Validation Standard, Chapter 1.1.6, Section A.2.1)**

112 ~~The WOA Validation Standard, Chapter 1.1.6 states that test methods and related procedures must be
113 appropriate for specific diagnostic applications in order for the test results to be of relevance. In other words,
114 the assay must be 'fit for purpose'. Many assays are developed with good intentions but without a specific
115 application in mind. At the very outset, it is critical that the diagnostic purpose(s) should be defined with respect
116 to the population(s) to be tested. The most common purposes are listed in broad terms in Section A of the
117 WOA Validation Standard, chapter 1.1.6. As such, they are inclusive of more narrow and specific
118 applications. However, these specific purpose(s) need to be clearly defined from the outset and are critically
119 important in the context of a fully validated assay. As will be seen in the following descriptions, clearly defining
120 the application will have impact on both the selection of reference samples and panels and the design of
121 analytical and diagnostic evaluations.~~

122 **2. Operating range (WOAH Validation Standard, Chapter 1.1.6, Section A.2.2–3) and 123 analytical sensitivity (WOAH Validation Standard, Chapter 1.1.6, Section B.1.3)**

124 **2.1. Analytical approaches Operating range and analytical sensitivity**

125 ~~The operating range of the assay is defines the lower and upper analyte detection limits and the
126 interval of analyte concentrations (amounts) over which the method provides suitable accuracy and
127 precision. It also defines the lower and upper detection limits the assay. To establish this range, The
128 operating range is established by serial dilution, to extinction, of replicates of a high-strong positive
129 reference sample is selected. This high positive sample, either natural or prepared, is serially diluted
130 to extinction. Dilutions of the strong positive are made in a negative matrix representative of the
131 typical sample matrix of samples type taken from animals in the population targeted by the assay.
132 This includes antibody assays where a high replicates of a strong positive reference serum should
133 be diluted in a negative reference serum to create the dilution series. Analytical sensitivity (ASe) is
134 measured by replicates of the lower limit of detection (LOD) of an analyte in an assay. The same
135 high-strong positive reference sample may be used to determine both the operating range and the
136 analytical LOD.~~

137 **2.2. Comparative approaches to analytical sensitivity**

138 If the intended purpose is to detect low levels of analyte or subclinical infections, it may be difficult to
139 obtain the appropriate reference materials from early stages of the infection process. In some cases,
140 it may be useful to determine a comparative ASe by running a panel of samples on the candidate

141 assay and on another independent assay. Ideally this panel of samples would be serially collected
142 from either naturally or experimentally infected animals and should represent infected animals early
143 after infection, ~~on~~ through to the development of clinical or fulminating disease, if possible. This would
144 provide a relative comparison of ASe between the assays, ~~as well as,~~ and a temporal comparison of
145 the earliest point of detection relative to the pathogenesis of the disease.

146 An experiment like the one described above, provides a unique opportunity to collect reference
147 samples representing a natural range of concentrations that would be useful for other validation
148 purposes. Care must be taken to avoid use of such samples when inappropriate (consult Group D
149 below). Wherever possible serial samples should be collected from ~~at least five~~ a statistically sound
150 number of animals throughout the course of infection. In cases where sampling is lethal (e.g.
151 requiring the harvest of internal organ tissues), the number of animals required ~~would be a minimum~~
152 depends on need and fitness of five per sampling event ~~the experimental approach.~~ In all cases
153 approval from an ethics committee is required. For smaller host species, ~~this~~ the number may need
154 to be increased in order to collect sufficient reference material. Given that experiments like this
155 require a high commitment of resources, it would be wise to maximise the collection of not only the
156 currently targeted reference samples but additional materials (e.g. multiple tissues, fluids, etc.) that
157 may be useful as reference materials in the future.

158 **3. Optimisation (WOAH Validation Standard, Chapter 1.1.6, Section A.2.32) and preliminary** 159 **repeatability (WOAH Validation Standard, Chapter 1.1.6, Section A.2.68)**

160 Optimisation is the process by which the most important physical, chemical and biological parameters of an
161 assay are evaluated and adjusted to ensure that the performance characteristics of the assay are best suited
162 to the intended application. At least three reference samples representing negative, low-weak and high-strong
163 positive may be chosen from either natural or prepared reference samples. Optimisation experiments are
164 rather exhaustive especially when assays with multiple preparatory and testing steps are involved. It is very
165 important that a sufficient quantity of each reference sample be available to complete all optimisation
166 experiments. Changing reference samples during the course of optimisation is not recommended as this will
167 result in the addition of an uncontrolled variable and a disruption in the continuity of optimisation evidence.

168 Assessment of repeatability should begin during assay development and optimisation stages. ~~Repeatability~~
169 and is further verified during Stage 1 of assay validation (Section B.1.1 of chapter 1.1.6). The same reference
170 samples should be used ~~for both processes, again~~ throughout to provide continuity of evidence.

171 **4. Calibration and process controls (WOAH Validation Standard, Chapter 1.1.6, Section** 172 **A.2.6)**

173 **4.1. International, national or in-house analyte reference standards**

174 International reference standards are highly characterised, contain defined concentrations of analyte,
175 and are usually prepared and held by international reference laboratories. They are the reagents to
176 which all assays and/or other reference materials should be standardised. National reference
177 standards are calibrated by comparison with an international standard reagent whenever possible.
178 In the absence of an international standard, a national reference standard may be selected or
179 prepared and it then becomes the standard of comparison for the candidate assay. In the absence
180 of both of the above, an in-house standard should be selected or prepared by the development
181 laboratory within the responsible organisation. In all cases, thorough documentation of reference
182 material should be observed as summarised in Figure 2. All of the standard reagents, whether natural
183 or prepared, must be highly characterised through extensive analysis, and preferably the methods
184 for their characterisation, preparation, and storage have been published in peer-reviewed
185 publications (Watson et al., 2021). These reference standards should also be both stable and
186 innocuous.

187 Reference standards, especially antibody, are usually provided in one of two formats. They may be
188 provided as a single positive reagent of given titre with the expectation that the candidate assay will
189 be standardised to give an equivalent titre. This is a straight forward analytical approach but many
190 of these 'single' standards have been prepared from highly positive samples as a pre-dilution in a

191 negative matrix in order to maximise the number of aliquots available. The drawback here is that
192 there is no accounting for any potential matrix effect in the candidate assay as there is no matrix
193 control provided. The other approach is to provide a negative and a low-weak and high-strong positive
194 set of reference standards that are of known concentrations or reactivities and are within the
195 operating range of the standard method that was used to prepare them. The negative provided in
196 the set must be the same as the negative diluent used to prepare the weak and strong positive
197 reference standard, if the positive standards were diluted. This compensates for any potentially
198 hidden matrix effect. In addition, this set of three acts as a template for the selection and/or
199 preparation of process controls (discussed below).

200 Classically, the above standards usually have been polyclonal antibody standards and to a lesser
201 extent, conventional antigen standards used for calibration of serological assays. However, today,
202 reference standards could also be monoclonal antibodies or recombinant/expressed proteins or
203 genomic constructs, if they are to be used to calibrate assays to a single performance standard.

204 **4.2. Working standards or process controls**

205 Working standard reagent(s), commonly known as quality or process controls, are calibrated to
206 international, national, or in-house standard reagents. They are selected or prepared in the local
207 matrix which is found in the population for which the assay is intended. Ideally, negative and low
208 weak and high-strong positive working standards should be selected or prepared. Concentrations
209 and/or reactivities should be within the normal operating range of the assay. Large quantities should
210 be prepared, aliquoted and stored for routine use in each diagnostic run of the assay. The intent is
211 that these controls should mimic, as closely as possible, field samples and should be handled and
212 tested like routine samples. They are used to establish upper and lower control limits of assay
213 performance and to monitor random and/or systematic variability using various control charting
214 methods. Their daily performance will determine whether or not an assay is in control and if individual
215 runs may be accepted. As such, these working reference samples are critically important from a
216 quality management standpoint.

217 **5. Technical modifications (~~WOAH Validation Standard, Chapter 1.1.6, Section B.5.2.1~~)**

218 Technical modifications to a validated assay such as changes in instrumentation, extraction protocols, and
219 conversion of an assay to a semi-automated or fully automated system using robotics will typically not
220 necessitate full revalidation of the assay. Rather, a methods comparison study may be done to determine if
221 these minor modifications to the assay protocol will affect the test results. Consult See chapter 2.2.8
222 Comparability of assays after changes in a validated test method for description of experiments and statistical
223 approaches to assay precision in the face of technical modifications that are appropriate for comparability
224 testing (Bowden & Wang, 2021; Reising *et al.*, 2021).

225 In general, these approaches require the use of three reference samples, a negative, a weak and a low and
226 high-strong positive. ~~Again these samples to represent the entire operating range of both assays. Samples~~
227 may be either natural or prepared. The important point to re-iterate here is that the same reference samples
228 that were used in the developmental stages of the assay may be used to assess modifications after the method
229 has been put into routine diagnostic use. This provides a higher level of confidence assessing potential impacts
230 because the performance characteristics of these reference samples have been well characterised. At the very
231 least, if new reference samples are to be used, they should be selected or prepared using the same criteria or
232 preparation procedures established for previous materials. ~~Again as~~ this enhances the continuity of evidence.

233 **6. Reagent replacement (~~WOAH Validation Standard, Chapter 1.1.6, Section B.5.2.3~~)**

234 When a reagent such as a process control sample is nearing depletion, it is essential to prepare and repeatedly
235 test a replacement before such a control is depleted. The prospective replacement should be included in
236 multiple runs of the assay in parallel with the original control to establish their proportional relationship. It is
237 important to change only one control reagent at a time to avoid the compound problem of evaluating more
238 than one variable.

239 ~~Again, it cannot be over-emphasised that any~~ Replacement reference reagent should be selected or prepared
240 using the same criteria or preparation procedures established for previous materials. ~~Again as~~ this enhances

241 the continuity of evidence and confidence in the assay and underlines the importance of documentation of
242 reference material data (Figure 2).

243

B. GROUP B

244 1. Analytical specificity (WOAH Validation Standard, Chapter 1.1.6, Section B.1.2)

245 Analytical specificity (ASp) is the degree to which the assay distinguishes between the target analyte and other
246 components that may be detected in the assay. ~~This is a relatively broad definition that is often not well~~
247 ~~understood. ASp may be broken down into different elements as described below.~~

248 The choice of reference samples that are required to assess ASp is highly dependent on the specific-intended
249 purpose or application that was originally envisaged defined at the development stage of the assay.
250 Assessment of ASp is a crucial element in proof of concept and verification of fitness for purpose and may be
251 broken down into three elements: selectivity, exclusivity and inclusivity.

252 Selectivity: an important element is the extent to which a method can accurately detect and or quantify the
253 targeted analyte in the milieu of nucleic acids, proteins and/or antibodies in the test matrix. This is sometimes
254 termed 'selectivity'. An example is the use of reference samples for tests that are designed to differentiate
255 infected from vaccinated animals (DIVA tests).

256 Reference samples need to be selected and tested from i) non-infected/non-vaccinated, ii) non-
257 infected/vaccinated, iii) infected/non-vaccinated, and iv) infected/vaccinated animals. These samples may be
258 collected under field conditions but it is important that an accurate history be collected, ideally with respect to
259 the animals, but at least to the herds involved, including vaccination practices and disease occurrences (Figure
260 2). Alternatively, it may be necessary to produce this material in experiments like those described in Section
261 A.2.2 of this chapter, ~~but~~ including a combination of experimentally vaccinated and challenged animals. ~~#~~
262 Application of the 3 Rs (replacement, reduction and refinement) aims to avoid or minimise the number of
263 animals used in experiments. For enzyme-linked immunosorbent assays (ELISAs), it is important to avoid use
264 of the vaccine as capture antigen in the assay (e.g. indirect ELISA enzyme-linked immunosorbent assay [I-
265 ELISA]), because carrier proteins in the vaccine may stimulate non-specific antibody responses in vaccinated
266 animals that may be detected in ELISA leading to false positives in the assay. Similarly to the comparative
267 approach described above with respect to ASe, at least five animals in each group should be considered. For
268 smaller host species, this number may need to be increased in order to collect sufficient reference material,
269 leading to false positives in the assay. Depending on the DIVA test, a single experiment could be designed to
270 assess aspects of both ASe and ASp.

271 ~~A second element, sometimes termed 'exclusivity',~~ Exclusivity is the capacity of the assay to detect an analyte
272 or genomic sequence that is unique to a targeted organism, and excludes all other other known organisms
273 that are potentially cross-reactive. This is especially true in serological assays where there are many examples
274 of antigens expressed by other organisms that are capable of eliciting cross-reacting antibody. An attempt
275 should be made to obtain reference samples from documented cases of infections ~~and/or~~ organisms that may
276 be cross-reactive. Depending on the type of assay, these reference materials may represent the organism
277 itself, host-derived samples, or genomic sequences. A profile for the exclusivity of the assay should be
278 established, and expanded on a continual basis as potentially cross-reactive organisms arise.

279 ~~Thirdly, a critical design consideration~~ Inclusivity relates to the capacity of an assay to detect one or several
280 strains or serovars of a species, several species of a genus, or a similar grouping of closely related ~~organisms~~
281 viruses, bacteria or antibodies. This defines the scope of detection and thus the fitness for purpose. Reference
282 samples are required to define the scope of the assay. If for example an assay is developed as a screening
283 test to detect all known genotypes or serotypes of a virus, then reference samples from each representative
284 type should be tested. As new lineages or serotype variants arise, they too should be tested as part of the test
285 profile, which should be updated on an ongoing basis.

286 **2. Analytical accuracy of adjunct ancillary tests (WOAH Validation Standard, Chapter 1.1.6,**
287 **Section B.1.4)**

288 Some test methods or procedures are solely analytical tools ~~and are usually applied~~ used to further
289 characterise an analyte that has been detected in a primary assay, ~~for example assays like~~ Examples are the
290 virus neutralisation tests used to type an isolated virus or characterise an antibody response and subtyping of
291 haemagglutinin genes by polymerase chain reaction of avian influenza virus. Such adjunct-ancillary tests must
292 be validated for analytical performance characteristics, ~~but and differ from~~ to routine diagnostic tests because
293 they do not require validation for diagnostic performance characteristics. The analytical accuracy of these tests
294 is often dependant on the use of reference ~~reagents-material~~. These reagents, whether they are antibody for
295 typing strains of organisms or reference strains of the organism, etc., should be thoroughly documented, as
296 required for any other reference material (Figure 2), with respect to their source, identity and performance
297 characteristics.

298 **C. GROUP C**

299 Reference samples in Group C may be used for a number of purposes. In the initial development stages, they
300 may be used in the assessment of assay repeatability and both preliminary reproducibility in Stage 1 and the
301 more in depth assessment of reproducibility in Stage 3 of the Validation Pathway. However, these samples
302 have a number of other potential uses once the assay is transferred to the diagnostic laboratory. They may be
303 used as panels for training and qualifying of analysts, and for assessing laboratory proficiency in external ring
304 testing programmes. Ideally, 20 or more individual samples should be prepared in large volumes. About a
305 quarter (25%) should be negative samples and the remainder (75%) should represent a collection of positives
306 spanning the operating range of the assay. They should be aliquoted into individual tubes in sufficient volumes
307 for single use only and stored for long term use (Chapter 1.1.2 Collection, submission and storage of diagnostic
308 specimens). The number of aliquots of each that will be required will depend on how many laboratories will be
309 using the assay on a routine diagnostic basis and how often proficiency testing is anticipated. Ideally, they
310 should be prepared in an inexhaustible quantity, but this is seldom feasible. At a minimum, several hundred or
311 more aliquots of each should be prepared at a time if the assay is intended for use in multiple laboratories.
312 This allows assessment of laboratory proficiency by testing the same sample over many testing intervals – a
313 useful means of detecting systematic error (bias) that may creep into long term use of an assay.

314 These samples may be natural or prepared from either single or pooled starting material. The intent is that
315 they should mimic as closely as possible a true test sample. Because mass storage is always a problem, it
316 may be necessary to store these materials in bulk and prepare working aliquots from time to time. However, if
317 storage space is available, it is preferable to prepare and store large numbers of aliquots at one time because
318 bulk quantities of analyte, undergoing freeze–thaw cycles to prepare a few aliquots at a time, may be subject
319 to degradation. Because this type of reference material is consumed at a fairly high rate, they will need to be
320 replaced or replenished on a continual basis. As potential replacement material is identified during routine
321 testing or during outbreaks, it is advisable to work with field counterparts to obtain bulk reference material and
322 store it for future use. Alternatively, it may be necessary to produce this material in experiments like those
323 described in Section A.2.2 of this chapter. Similar to the comparative approach described above with respect
324 to ASe, at least five animals in each group should be considered. For smaller host species, this number may
325 need to be increased in order to collect sufficient reference material.

326 **1. Repeatability (WOAH Validation Standard, Chapter 1.1.6, Section B.1.1) and preliminary**
327 **reproducibility-provisional assay recognition (WOAH Validation Standard, Chapter 1.1.6,**
328 **Section B.2.6)**

329 Repeatability is the level of agreement between results of replicates of a sample both within and between runs
330 of the same test method in a given laboratory. Repeatability is estimated by evaluating variation in results of
331 replicates from a minimum of three (preferably five) samples representing analyte activity within the operating
332 range of the assay. Consult Chapter 2.2.4 *Measurement uncertainty* for statistical approaches for measures
333 of uncertainty for assessments of repeatability.

334 Reproducibility is the ability of a test method to provide consistent results, as determined by estimates of
335 precision, when applied to aliquots of the same samples tested in different laboratories. However, preliminary

336 reproducibility estimates of the candidate assay should be determined during developmental stages. A small
337 panel of three (but preferably five) representing negative, weak and ~~both low and high~~ strong positives, like
338 those described above, would be adequate. This type of panel could also be used for a limited evaluation of
339 reproducibility to enhance provisional acceptance status for the assay. The test method is usually assessed in
340 ~~one~~ two or more laboratories with a high level of experience and proficiency in assays similar to the candidate
341 assay. The panel of 'blind' samples is evaluated using the candidate assay in each of these laboratories, using
342 the same protocol, same reagents and comparable equipment. This is a scaled-down version of Stage 3 of
343 assay validation. ~~Consult Chapter 2.2.4 for further explanation of the topic and its application.~~

344 **2. Reproducibility (WOAH Validation Standard, Chapter 1.1.6, Section B.3)**

345 Reproducibility is an important measure of the precision of an assay when used in a cross-section of
346 laboratories located in distinct or different regions or countries using the identical assay (protocol, reagents
347 and controls). As the number of laboratories increases, so does the number of variables encountered with
348 respect to laboratory environments, equipment differences and technical expertise. ~~These~~ An overview of the
349 factors affecting testing reproducibility is provided in Waugh & Clark (2021). Reproducibility studies are a
350 measure of an assay's capacity to remain unaffected by substantial changes or substitutions in test conditions
351 anticipated in multi-laboratory use (e.g. shipping conditions, technology transfer, reagents batches, equipment,
352 testing platforms and/or environments). ~~Each of~~ At least three laboratories should test the same panel of 'blind'
353 samples containing a minimum of 20 samples, representing negative and a range of positive samples. If
354 selected negative and/or positive samples in the panel are duplicated, in the panel then it may be possible to
355 assess both assay reproducibility and within-laboratory repeatability ~~estimates may be augmented by replicate~~
356 ~~testing of these samples when used in the reproducibility studies.~~

357 **3. Proficiency testing (WOAH Validation Standard, Chapter 1.1.6, Section B.5.1)**

358 A validated assay in routine use in multiple laboratories needs to be continually monitored to ensure uniform
359 performance and provide overall confidence in test results. This is assessed through external quality assurance
360 programmes. Proficiency testing is one measure of laboratory competence derived by means of an inter-
361 laboratory comparison; implied is that participating laboratories are using the same (or similar) test methods,
362 reagents and controls. Results are usually expressed qualitatively, i.e. either negative or positive, to determine
363 pass/fail criteria. However, ~~for single dilution assays, where~~ semi-quantitative results provide are provided,
364 additional data for assessment of analysis may assess non-random error among the participating laboratories.
365 Refer to Johnson & Cabuang (2021) for an overview of proficiency testing and ring trials.

366 Proficiency testing programmes are varied depending on the type of assay in use. For single dilution type
367 assays, panel sizes also vary but a minimum of five samples, representing negative and both low and high
368 positives, like those described above, would be adequate. Proficiency testing is not unlike a continuous form
369 of reproducibility assessment. However, reproducibility, by definition, is a measure of the assay's performance
370 in multiple laboratories; whereas proficiency testing is an assessment of laboratory competence in the
371 performance of an established and validated assay. Measurements of precision can be estimated for both the
372 reproducibility and repeatability data if replicates of the same reference sample are included in this 'blind'
373 panel. ~~Consult Chapter 2.2.4 for further explanation of the topic and its application.~~ vary but a minimum of five
374 samples, representing negative weak and strong positives, would be adequate.

375 **D. GROUP D**

376 Reference samples in Group D differ from the previous Groups in that each sample in the panel should be
377 from a different individual animal. As indicated in Chapter 2.2.8 Comparability of assays after changes in a
378 validated test method, experimental challenge studies often include repeated sampling of individual animals
379 to determine the progression of disease, but this is a different objective ~~than~~ to comparing performance
380 characteristics that would be associated with diagnostic sensitivity (DSe) and diagnostic specificity (DSp) of a
381 test method. Serially drawn samples, taken on different days from the same animal, cannot be used as
382 representative of individual animals in populations targeted by the assay, because such samples violate the
383 rule of independence of samples required for such studies.

384 Care must be taken in choosing the reference samples and the standard (independent) method used in this
385 type of comparison to ensure that the analytes being detected (if different) demonstrate the same type of
386 pathogenic profile in terms of time of appearance after exposure to the infectious agent, and relative
387 abundance in the test samples chosen.

388 **1. Standard method comparison and provisional recognition (~~WOAH Validation Standard,~~**
389 **Chapter 1.1.6, Sections B.2.6-5 and B.2.6)**

390 There are situations where it is not possible or desirable to fulfil Stage 2 of the Validation Pathway because
391 appropriate samples from the target population are scarce and animals are difficult to access (such as for
392 exotic diseases). However, a small but select panel of highly characterised test samples representing the
393 range of analyte concentration should be run in parallel in the candidate assay method and ~~by a~~ WOA
394 standard method, as published in the WOA *Manuals*. Biobanks may be a useful resource in this context,
395 providing well-characterised samples supported with metadata to enhance transparency and provenance of
396 samples used in method comparisons (Watson *et al.*, 2021). If the methods are deemed to be comparable
397 (Chapter 2.2.8), and depending on the intended application of the assay, the choice may be made that further
398 diagnostic validation is not required. For example, if the intended application is for screening of imported
399 animals or animal products for exotic pathogens or confirmation of clinical signs, full validation beyond a test
400 method comparison may not be feasible or warranted.

401 Experience has shown that the greatest obstacle to continuing through Stage 2 of the Validation Pathway is
402 the number of defined samples required to estimate diagnostic performance parameters with a high degree of
403 certainty (~~WOAH Validation Standard, chapter 1.1.6, Section B.2~~). In some cases, provisional recognition by
404 international, national or local authorities may be granted for an assay that has not been completely evaluated
405 past analytical stages. The different rationales for provisional acceptance are well explained in ~~the WOA~~
406 Validation Standard, chapter 1.1.6. In all cases however, sound evidence must exist for comparative estimates
407 of DSp and DSe based on a small select panel of well-characterised samples containing the targeted analyte.

408 Ideally, for both comparison with a standard method or provisional recognition, a panel of, for example,
409 60 samples could be assembled to ensure sufficient sample size for statistical analysis of the resulting data.
410 This would include 30 'true' negatives and 30 'true' positives. Wherever possible, the positives should reflect
411 the range of analyte concentrations or activities expected in the target population. As mentioned above, each
412 sample in this panel must represent an individual animal. Consult Chapter 2.2.5 for statistical approaches to
413 determining methods comparability using diagnostic samples.

414 **2. Biological modifications (~~WOAH Validation Standard, Chapter 1.1.6, Section B.5.2.2~~)**

415 There may be situations where changes to some of the biologicals used in the assay may be necessary and/or
416 warranted. This may include changes to reagents themselves or a change to a different type of specimen
417 which contains the same analyte as targeted in the original validated assay (e.g. from serum to saliva). At the
418 very least, all of the analytical criteria of the validation pathway must be re-assessed before proceeding. If the
419 analytical requisites are met, the remaining question relates to whether or not a full diagnostic validation is
420 required. A similar approach to the above using a panel of 60 individual reference samples may be considered.
421 However, in this case the original test method would be considered as the standard (independent) test and the
422 modified method would be considered the candidate. Consult Chapter 2.2.5 for statistical approaches to
423 determining methods comparability using diagnostic samples.

424 **E. GROUP E**

425 Reference animals and reference samples in this Group E are well described in ~~the WOA~~
426 Standard, chapter 1.1.6, Section B.2.1). However, there are a few points that are worth re-iterating here.

427 **1. ‘Gold standard’² – diagnostic specificity and diagnostic sensitivity (WOAH Validation**
428 **Standard, Chapter 1.1.6, Section B.2.1)**

429 For conventional estimates of DSp, negative reference samples refer to true negative samples, from animals
430 that have had no possible infection or exposure to the agent. In some situations, where the disease has never
431 been reported in a country or limited to certain regions of a country, identification of true negative reference
432 samples is usually not a problem. However, where the disease is endemic, samples such as these may be
433 difficult to locate. It is often possible to obtain these samples from regions within a large country or perhaps
434 different countries where the disease in question does not occur or has either been eradicated or has never
435 had the disease in question.

436 ~~Again~~ For conventional estimates of DSe, positive reference samples refer to true positives. Care must be
437 taken to ensure that the sample population is representative of the population that will be the target of the
438 validated assay. It is generally problematic to find sufficient numbers of true positive reference animals, as
439 determined by isolation of the organism. It may be necessary to resort to samples from animals that have been
440 tested by a combination of methods that unequivocally classify animals as infected/exposed as discussed in
441 ~~the WOA Validation Standard, chapter 1.1.6.~~

442 ~~The important point here is that~~ All samples, irrespective of origin, must be documented as they would for any
443 other reference sample ~~so as to~~ unequivocally ~~to~~ classify animals as infected or exposed, dependent on the
444 fitness for purpose and proposed use of the test. As mentioned in Section A, and summarised in Figure 2, of
445 this chapter, all reference samples should be well characterised. ~~This includes documentation on both the~~
446 ~~pathogen and donor host. For pathogens, this may include details related~~ and data documented to strain,
447 serotype, genotype, lineage, etc. The source of the host material should be well described with respect to
448 species, breed, age, sex, reproductive status, vaccination history, herd history, etc. Wherever possible, the
449 phase of infection should be noted. This could include details related clinical signs, antibody profiles, pathogen
450 load or shedding, etc. In some cases, experimental infection/exposure may be the only viable option ensure
451 appropriate sample selection for the production of reference material (see the OIE Validation Standard, Section
452 B.2.3). In this case, all of the above and the experimental protocol should be detailed intended purpose.

453 Particularly relevant to these reference samples, the tests that are used to determine their so called ‘true’
454 disease/infection status need to be well documented in order to assess potential errors in estimates that may
455 be carried over into the estimates for the candidate assay. Indeed, when using imperfect standard assays to
456 define reference animal or sample status, the DSe and DSp performance estimates of the candidate assay
457 may be flawed and often overestimated. Consult Chapter 2.2.5 for statistical considerations. Situations where
458 a perfect reference is available for either positive or negative animals, and one where the reference is perfect
459 for both are described for diagnostic test validation by Heuer & Stevenson (2021).

460 **F. GROUP F**

461 **1. Animals of unknown status – diagnostic specificity and diagnostic sensitivity (WOAH**
462 **Validation Standard, Chapter 1.1.6, Section B.2.2)**

463 Latent-class models are introduced in ~~the WOA Validation Standard, chapter 1.1.6.~~ They do not rely on the
464 assumption of a perfect reference (standard or independent) test but rather estimate the accuracy of the
465 candidate test and the reference standard with the combined test results. Because these statistical models are
466 complex and require critical assumptions, statistical assistance should be sought to help guide the analysis
467 and describe the sampling from the target population(s), the characteristics of other tests included in the
468 analysis, the appropriate choice of model and the estimation methods based on peer-reviewed literature.
469 Consult Chapter 2.2.5 for statistical considerations.

470 Reference populations, not individual reference samples, used in latent-class studies need to be well
471 described. ~~This includes documentation on both the pathogen and donor host. For pathogens, this may include~~
472 ~~details related to strain, serotype, genotype, lineage, etc., that may be circulating in the population. The source~~
473 ~~of the host material should be well described with respect to species, breed, age, sex, reproductive status,~~

² The term “Gold Standard” is limited to a perfect reference standard as described in the WOA Validation Standard, chapter 1.1.6, Section B.2.1.2, and Chapter 2.2.5 *Statistical approaches to validation*, Introduction and Figure 1.

474 vaccination history, herd history, etc. as summarised in Figure 2. Wherever possible, the phase of infection in
475 the populations should be noted with respect to morbidity or mortality events, recovery, etc.

476 As a special note, if latent class models are to be used to ascribe estimates of DSe and DSp and include
477 multiple laboratories in the design, it is possible to incorporate an assessment of reproducibility into the
478 assessment. ~~As stated above, statistical advice should be sought in this respect.~~ Bayesian analysis of latent
479 class models are complex and require adherence to critical assumptions. Statistical assistance should be
480 sought to help guide the analysis and describe the sampling from the target population(s), the characteristics
481 of other tests included in the analysis, the appropriate choice of model and the estimation methods (based on
482 peer-reviewed literature). See chapter 2.2.5 for details and Cheung *et al.*, 2021.

483 **FURTHER READING**

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502 * *

503 **NB: There is a WOAHA Collaborating Centre for**
504 **Diagnostic Test Validation Science in the Asia-Pacific Region (please consult the WOAHA Web site:**
505 **<https://www.woah.org/en/what-we-offer/expertise-network/collaborating-centres/#ui-id-3>).**
506 **Please contact the WOAHA Collaborating Centre for any further information on validation.**

507 **NB: FIRST ADOPTED IN 2014.**

Annexe 8. Chapter 3.1.5. 'Crimean–Congo haemorrhagic fever'

CHAPTER 3.1.5.

CRIMEAN–CONGO HAEMORRHAGIC FEVER

SUMMARY

Crimean–Congo haemorrhagic fever virus (CCHFV) of the genus Orthonairovirus of the family Nairoviridae causes a zoonotic disease in many countries of Asia, Africa, the Middle East and south-eastern Europe. As the distribution of CCHFV coincides with the distribution of its main vector, ticks of the genus Hyalomma, the spread of infected ticks into new, unaffected areas facilitates the spread of the virus. The virus circulates in a tick–vertebrate–tick cycle, but can also be transmitted horizontally and vertically within the tick population. Hyalomma ticks infest a wide spectrum of different wildlife species, e.g. deer and hares, and free-ranging livestock animals, e.g. goat, cattle, and sheep. Many birds are resistant to infection, but ostriches appear to be more susceptible. Viraemia in livestock is short-lived, and of low intensity. These animals play a crucial role in the life cycle of ticks, and in the transmission and amplification of the virus and are, therefore, in the focus of veterinary public health. As animals do not develop clinical signs, CCHFV infections have no effect on the economic burden regarding livestock animal production. In contrast to animals, infections of humans can result in the development of a severe disease, Crimean–Congo haemorrhagic fever (CCHF).

Every year, more than 1000 human CCHF cases are reported with case fatality rates of 5–80% depending on the virus strain and other local factors. The pathogenesis of the disease in humans is not well understood. Most people become infected by tick bites and by crushing infected ticks, but infection is also possible through contact with blood and other body fluids of viraemic animals, for example in slaughterhouses. As CCHFV also has the potential to be transmitted directly from human-to-human, nosocomial outbreaks have been reported.

There is no approved CCHF vaccine available and therapy is restricted to treatment of the symptoms. Health education and information on prevention and behavioural measures are most important in order to enhance public risk perception and, therefore, decrease the probability of infections. Thus the identification of endemic areas is crucial for focused and targeted implementation of public health measures. Serological screening of ruminants allows CCHFV-affected areas to be identified, as antibody prevalence in animals is a good indicator of local virus circulation. Treatment with tick repellents can be quite effective in reducing the tick infestation of animals. To protect laboratory staff, handling of CCHFV infectious materials should only be carried out at an appropriate biocontainment level.

Detection and identification of agent: *Only a single virus serotype is known to date although sequencing analysis indicates considerable genetic diversity. CCHFV has morphological and physiochemical properties typical of the family Nairoviridae. The virus has a single-stranded, negative-sense RNA genome consisting of three segments: L (large), M (medium) and S (small), each of which is contained in a separate nucleocapsid within the virion. The virus can be isolated from serum or plasma samples collected during the febrile or viraemic stage of infection, or from liver of infected animals. Primary isolations are made by inoculation of several tissue cultures, commonly African green monkey kidney (Vero) cells. For identification and characterisation of the virus, conventional and real-time reverse transcription polymerase chain reaction (PCR) can be used. As infections of animals remain clinically unapparent, the likelihood of isolating virus from a viraemic animal is very low.*

44 **Serological tests:** Type-specific antibodies are demonstrable by indirect immunofluorescence
45 test or by IgG-sandwich and IgM-capture enzyme-linked immunosorbent assay. Commercial test
46 systems are available for animal health; in addition a few in-house systems have been published
47 or kits are used replacing the conjugate provided in kit with one that is suitable for the animal
48 species to be screened for CCHFV-specific antibodies.

49 **Requirements for vaccines:** There is no vaccine available for animals.

50 A. INTRODUCTION

51 Crimean-Congo haemorrhagic fever (CCHF) is a zoonotic disease caused by a primarily tick-borne CCHF
52 virus (CCHFV) of the genus *Orthonairovirus* of the family *Nairoviridae*, order *Bunyavirales*. CCHFV possesses
53 a negative-sense RNA genome consisting of three segments, L (large), M (medium) and S (small) each
54 contained in a separate nucleocapsid within the virion. All orthonairoviruses are believed to be transmitted by
55 either ixodid or argasid ticks, and only three are known to be pathogenic to humans, namely CCHF, Dugbe
56 and Nairobi sheep disease viruses (Swanepoel & Burt, 2004; Swanepoel & Paweska, 2011; Whitehouse,
57 2004). CCHFV can be grown in several tick cell lines derived from both a natural vector (*Hyalomma anatolicum*)
58 and other tick species not implicated in natural transmission of the virus (Bell-Sakyiet *et al.*, 2012).

59 The virus from an outbreak of “Crimean haemorrhagic fever” in the Crimean Peninsula in 1944 was not isolated
60 or characterised until 1967. “Congo haemorrhagic fever” virus, isolated from a patient in the former Zaire (now
61 Democratic Republic of the Congo) in 1956, was shown in 1969 to be the same virus. As a consequence the
62 names of both countries have been used in combination to describe the disease (Hoogstraal, 1979).
63 Distribution of the virus reflects the broad distribution of *Hyalomma* ticks, the predominant vector of the virus
64 (Avsic-Zupanc, 2007; Grard *et al.*, 2011; Papa *et al.*, 2011; Swanepoel & Paweska, 2011).

65 The natural cycle of CCHFV includes transovarial and transstadial transmission among ticks and a tick-
66 vertebrate-tick cycle involving a variety of wild and domestic animals. Infection can also be transferred between
67 infected and uninfected ticks during co-feeding on a host; so called ‘non-viraemic transmission’ phenomenon.
68 *Hyalomma* ticks feed on a variety of domestic ruminants (sheep, goats, and cattle), and wild herbivores, hares,
69 hedgehogs, and certain rodents. CCHFV infection in animals was reviewed by Nalca & Whitehouse (2007).
70 Experimental infections of wild animals and livestock with CCHFV were reviewed by Spengler *et al.* (2016).
71 Although animal infections are generally subclinical, the associated viraemia levels are sufficient to enable
72 virus transmission to uninfected ticks (Swanepoel & Burt, 2004; Swanepoel & Paweska, 2011). Many birds are
73 resistant to infection, but ostriches appear to be more susceptible than other bird species (Swanepoel *et al.*,
74 1998). Although they do not appear to become viraemic, ground feeding birds may act as a vehicle for spread
75 of CCHFV infected ticks. Results from serological surveys conducted in Africa and Eurasia indicate extensive
76 circulation of the virus in livestock and wild vertebrates (Swanepoel & Burt, 2004).


77 Humans acquire infection from tick bites, or from contact with infected blood or tissues from livestock or human
78 patients. After incubation humans can develop a severe disease with a prehaemorrhagic phase, a
79 haemorrhagic phase, and a convalescence period. Haemorrhagic manifestations can range from petechiae to
80 large haematomas. Bleeding can be observed in the nose, gastrointestinal system, uterus and urinary tract,
81 and the respiratory tract, with a case fatality rate ranging from 5% to 80% (Ergonul, 2006; Yen *et al.*, 1985;
82 Yilmaz *et al.*, 2008). The severity of CCHF in humans highlights the impact of this zoonotic disease on public
83 health. Although CCHFV has no economic impact on livestock animal production, the serological screening of
84 animal serum samples for CCHFV-specific antibodies is very important. As seroprevalence in animals is a
85 good indicator for local virus circulation, such investigations allow identification of high-risk areas for human
86 infection (Mertens *et al.*, 2013). Slaughterhouse workers, veterinarians, stockmen and others involved with the
87 livestock industry should be made aware of the disease. They should take practical steps to limit or avoid
88 exposure of naked skin to fresh blood and other animal tissues, and to avoid tick bites and handling ticks.
89 Experiences from South Africa demonstrated that the use of repellents on animals before slaughter could
90 reduce the numbers of infected slaughterhouse workers (Swanepoel *et al.*, 1998). The treatment of livestock
91 in general can reduce the tick density among these animals and thus reduce the risk of tick bite in animal
92 handlers (Mertens *et al.*, 2013). Such tick control by the use of acaricides is possible to some extent, but may
93 be difficult to implement under extensive farming conditions. Inactivated mouse brain vaccine for the prevention
94 of human infection has been used on a limited scale in Eastern Europe and the former USSR (Swanepoel &

95 Paweska, 2011). Progress in CCHFV vaccine development is being made with several different approaches
 96 trialled to overcome current challenges (Dowall *et al.*, 2017).

97 Infectivity of CCHFV is destroyed by boiling or autoclaving and low concentrations of formalin or beta-
 98 propriolactone. The virus is sensitive to lipid solvents. It is labile in infected tissues after death, presumably
 99 due to a fall in pH, but infectivity is retained for a few days at ambient temperature in serum, and for up to 3
 100 weeks at 4°C. Infectivity is stable at temperatures below –60°C (Swanepoel & Paweska, 2011). CCHFV should
 101 be handled with appropriate biocontainment measures determined by risk analysis as described in Chapter
 102 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal*
 103 *facilities* (Palmer, 2011; Whitehouse, 2004).

104 B. DIAGNOSTIC TECHNIQUES

105 **Table 1.** Diagnostic test formats for Crimean-Congo haemorrhagic fever virus infections in animals

Method	Purpose					
	Populatio n freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmatio n of clinical cases in animals	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection and identification of the agent^(a)						
Real-time RT-PCR	–	++ 	–	+++ ^(b)	+ ^(c)	–
Virus isolation in cell culture	–	–	–	+ ^(b)	–	–
Detection of immune response						
IgG ELISA	+++	+	–	++ ^(d)	+++	–
Competitive ELISA	+++	+	–	++ ^(d)	+++	–
IgM ELISA	–	++	–	++ ^(e)	–	–

106 Key: +++ = recommended for this purpose; ++ recommended but has limitations;
 107 + = suitable in very limited circumstances; – = not appropriate for this purpose.

108 RT-PCR = reverse-transcription polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay.

109 ^(a)A combination of agent identification methods applied on the same clinical sample is recommended.

110 ^(b)Molecular testing/isolation can be used to confirm acute infection in rare cases in animals showing
 111 clinical signs as viraemia tends to be transient.

112 ^(c)RT-PCR is used for the screening of tick populations in the context of surveillance studies.

113 ^(d)Serological evidence of active infection with CCHFV has been demonstrated by seroconversion based on a rise in total or IgG
 114 antibody titres on samples taken at 2–4 weeks apart.

115 ^(e)Serological evidence of active infection with CCHFV has been demonstrated by the detection of IgM antibodies specific to

116 CCHFV infection causes only a mild fever in domestic and wild vertebrate animals with a detectable viraemia
 117 of up to 2 weeks (Gonzalez *et al.*, 1998; Gunes *et al.*, 2011). Similarly infected ostriches develop only low and
 118 short-lived viraemia and no clinical signs (Swanepoel & Burt, 2004). Therefore, recent infections in animals
 119 are rarely diagnosed and methods such as polymerase chain reaction (PCR), virus isolation in cell culture and
 120 IgM detection by enzyme-linked immunosorbent assay (ELISA) are mainly used in human CCHF diagnostics
 121 or in the special case that an animal has to be classified as CCHFV free. For prevalence analysis and for
 122 determination of whether CCHFV is circulating in a country, methods for the detection of IgG antibodies are
 123 preferred (Table 1). If there is any possibility or suspicion that diagnostic samples could be contaminated with
 124 CCHFV, they should be handled under an adequate biosafety level and all persons dealing with those samples
 125 should be aware of the possible risk and should use personal protective equipment to avoid human infections.

126 **1. Detection and identification of the agent**

127 For testing animals for viraemia, rapid diagnosis can be achieved by detection of viral nucleic acid in serum or
128 plasma using conventional (Burt *et al.*, 1998) or real-time reverse transcription (RT-) PCR (Drosten *et al.*, 2002;
129 Duh *et al.*, 2006; Koehler *et al.*, 2018; Negredo *et al.*, 2017; Sas *et al.*, 2018; Wolfel *et al.*, 2007), or by
130 demonstration of viral antigen (Shepherd *et al.*, 1988). Specimens to be submitted for laboratory confirmation
131 of CCHF include blood and liver samples. Because of the risk of laboratory-acquired infections, work with
132 CCHFV should be conducted in appropriate biosafety facilities.

133 The virus can be isolated from serum and organ suspensions in a wide variety of cell cultures, including Vero,
134 LLC-MK2, SW-13, BSR-T7/5, CER and BHK21 cells, and identified by immunofluorescence using specific
135 antibodies. Isolation and identification of virus can be achieved in 1–5 days, but cell cultures lack sensitivity
136 and usually only detect high concentrations of virus present in the blood.

137 **1.1. Virus isolation in cell culture**

138 CCHFV can be isolated in mammalian cell cultures. Vero cells are commonly used, usually yielding
139 an isolate between 1 and 5 days post-inoculation (p.i). CCHFV is poorly cytopathic and thus infectivity
140 is titrated by demonstration of immunofluorescence in infected cells (Shepherd *et al.*, 1986). SW-13
141 cell line has also been used extensively for virus isolation, producing plaques within 4 days (p.i.).
142 Identification of a CCHFV isolate has to be confirmed by immunofluorescence or molecular
143 techniques (Burt *et al.*, 1998; Shepherd *et al.*, 1986).

144 **1.1.1. Test procedure**

- 145 i) Susceptible cell lines include Vero-E6, BHK-21, LLC-MK2 and SW-13 cells. Inoculate
146 80% confluent monolayers of the preferred cell line with the specimen. The volume of
147 specimen to be used depends on the size of the culture vessel (i.e. 25 cm² culture flask
148 or 6- or 24-well tissue culture plate). The specimen volume should be sufficient to cover
149 the cell monolayer. Samples of insufficient volumes can be diluted with tissue culture
150 medium to prepare sufficient inoculation volume.
- 151 ii) Adsorb the specimen for 1 hour at 37°C.
- 152 iii) Remove inoculum. Add fresh tissue culture medium containing 2% fetal calf serum and
153 other required additives, as per specific medium and cell line requirements.
- 154 iv) Incubate at 37°C and 5% CO₂ for 4–7 days.
- 155 v) Test supernatant for presence of CCHFV viral RNA using real-time RT-PCR as described
156 below, or perform immunofluorescence assay on cell scrapings.
- 157 vi) Isolates of CCHFV from clinical specimens cause no microscopically recognisable
158 cytopathic effects (CPE) in most of these cell lines.

159 **1.2. Nucleic acid detection**

160 Molecular-based diagnostic assays, such as RT-PCR, serve as the front-line tool in the diagnosis of
161 CCHF, as well as other viral haemorrhagic fevers (Drosten *et al.*, 2003). The benefit of molecular
162 diagnostic assays is their rapidity compared to virus culture, often allowing a presumptive diagnosis to
163 be reported within a few hours after receiving a specimen (Burt *et al.*, 1998). The RT-PCR is a sensitive
164 method for diagnosis, but because of the genetic diversity of CCHFV, there might be some challenges
165 with regard to design of primers or probes that allow detection of all circulating strains of the virus.
166 Indeed, based on geographical origin and phylogenetic analyses of the S gene segment, CCHFV has
167 previously been classified into nine geographical clades – four predominantly diffused in Africa, three
168 in Europe, and two in Asia. Several real-time RT-PCR assays that detect strains from different
169 geographical locations have been evaluated (Gruber *et al.*, 2019). While some assays have been
170 shown to be highly sensitive, detecting as little as 10 viral RNA copies per ml of plasma, it is necessary
171 to combine at least two molecular assays to ensure detection of the different CCHFV clades (Gruber
172 *et al.*, 2019). The best assay combination(s) with the best detection efficacy for each CCHFV clade,
173 on the basis of all CCHFV sequences known at the time of the study, are shown in Table 2. In
174 addition, a low-density microarray has been extensively validated in clinical specimens collected from

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confirmed cases of CCHF over 20 years by a WHO reference laboratory. It was shown to detect as few as 6.3 genome copies per reaction (Wolfel *et al.*, 2009).

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Table 2. Molecular assay combinations for the detection of CCHFV-specific nucleic acid

Clade	Molecular assay combinations	Primer and probe names (5' → 3' sequence)
Africa 1	Real-time RT-PCR	Fwd CCRRealP1 (TCT-TYG-CHG-ATG-AYT-CHT-TYC) Rev CCRRealP2 (GGG-ATK-GTY-CCR-AAG-CA) Probe (ACA-SRA-TCT-AYA-TGC-AYC-CTG-C)
Africa 2	Real-time RT-PCR	Fwd CCRRealP1 (TCT-TYG-CHG-ATG-AYT-CHT-TYC) Rev CCRRealP2 (GGG-ATK-GTY-CCR-AAG-CA) Probe (ACA-SRA-TCT-AYA-TGC-AYC-CTG-C)
	Real-time RT-PCR	Fwd CCHF-SF2 (GGA-VTG-GTG-VAG-GGA-RTT-TG) Rev CCHF-SR2 (CAD-GGT-GGR-TTG-AAR-GC) Fwd CCHF-N2 (CAA-RGG-CAA-RTA-CAT-MAT)
Africa 3	Nested RT-PCR	Fwd CCHF1 (CTG-CTC-TGG-TGG-AGG-CAA-CAA) Rev CCHF2_5 (TGG-GTT-GAA-GGC-CAT-GAT-GTA-T) Nested Fwd CCHFn15 (AGG-TTT-CCG-TGT-CAA-TGC-AAA) Nested Rev CCHFn25 (TTG-ACA-AAC-TCC-CTG-CAC-CAG-T)
	Nested RT-PCR	Fwd CrCon1+ (RWA-AYG-GRC-TTR-TGG-AYA-CYT-TCA-C) Rev CrCon1- (TRG-CAA-GRC-CKG-TWG-CRA-CWA-GWG-C) Nested Fwd CriCon2+ (ART-GGA-GRA-ARG-AYA-TWG-GYT-TYC-G) Nested Rev CriCon2- (CYT-TGA-YRA-AYT-CYC-TRC-ACC-ABT-C)
Africa 4	Real-time RT-PCR	Fwd CCRRealP1 (TCT-TYG-CHG-ATG-AYT-CHT-TYC) Rev CCRRealP2 (GGG-ATK-GTY-CCR-AAG-CA) Probe (ACA-SRA-TCT-AYA-TGC-AYC-CTG-C)
	Real-time RT-PCR	Fwd CCHF-III (CAA-GAG-GTA-CCA-AGA-AAA-TGA-AGA-AGG-C) Rev CCHF-III-r (GCC-ACG-GGG-ATT-GTC-CCA-AAG-CAG-AC) Probe CCHFprobe-1 (ATC-TAC-ATG-CAC-CCT-GCY-GTG-YTG-ACA) Probe CCHFprobe-2 (TTC-TTC-CCC-CAC-TTC-ATT-GGR-GTG-CTC-A)
Asia 1	Nested PCR	Fwd CCF-115F (AAR-GGA-AAT-GGA-CTT-RTG-GA) Fwd CCF-131F (TGG-AYA-CYT-TCA-CAA-ACT-CC) Rev CCF-759R (GCA-AGG-CCT-GTW-GCR-ACA-AGT-GC)
	Real-time RT-PCR	Fwd CC1a_for (GTG-CCA-CTG-ATG-ATG-CAC-AAA-AGG-ATT-CCA-TCT) Rev CC1a_rev (GTG-CCA-CTG-ATG-ATG-CAC-AAA-AGG-ATT-CCA-TCT) Probe CCHF-01 (CAA-CAG-GCT-GCT-CTC-AAG-TGG-AG)
	Real-time RT-PCR	Fwd CCHF-SF2 (GGA-VTG-GTG-VAG-GGA-RTT-TG) Rev CCHF-SR2 (CAD-GGT-GGR-TTG-AAR-GC) Probe CCHF-N2 (CAA-RGG-CAA-RTA-CAT-MAT)
Asia 2	Nested PCR	Fwd CCF-115F (AAR-GGA-AAT-GGA-CTT-RTG-GA) Fwd CCF-131F (TGG-AYA-CYT-TCA-CAA-ACT-CC) Rev CCF-759R (GCA-AGG-CCT-GTW-GCR-ACA-AGT-GC)
	Sybrgreen Real-time RT-PCR	Fwd (GAT-GAG-ATG-AAC-AAG-TGG-TTT-GAA-GA) Rev (GTA-GAT-GGA-ATC-CTT-TTG-TGC-ATC-AT)
Europe 1	RT-PCR	Fwd CCS (ATG-CAG-GAA-CCA-TTA-ART-CTT-GGG-A) Rev 1 CCAS1 (CTA-ATC-ATA-TCT-GAC-AAC-ATT-TC) Rev 2 CCAS2 (CTA-ATC-ATG-TCT-GAC-AGC-ATC-TC)
	Real-time RT-PCR	Fwd CCRRealP1 (TCT-TYG-CHG-ATG-AYT-CHT-TYC) Rev CCRRealP2 (GGG-ATK-GTY-CCR-AAG-CA) Probe (ACA-SRA-TCT-AYA-TGC-AYC-CTG-C)
	Nested RT-PCR	Fwd CCF-115F (AAR-GGA-AAT-GGA-CTT-RTG-GA)

Clade	Molecular assay combinations	Primer and probe names (5' → 3' sequence)
		Fwd CCF-131F (TGG-AYA-CYT-TCA-CAA-ACT-CC) Rev CCF-759R (GCA-AGG-CCT-GTW-GCR-ACA-AGT-GC)
Europe 2	Nested RT-PCR	Fwd CrCon1+ (RWA-AYG-GRC-TTR-TGG-AYA-CYT-TCA-C) Rev CrCon1- (TRG-CAA-GRC-CKG-TWG-CRA-CWA-GWG-C) Fwd CriCon2+ (ART-GGA-GRA-ARG-AYA-TWG-GYT-TYC-G) Rev CriCon2- (CYT-TGA-YRA-AYT-CYC-TRC-ACC-ABT-C)
Europe 3	Real-time RT-PCR	Fwd CCRRealP1 (TCT-TYG-CHG-ATG-AYT-CHT-TYC) Rev CCRRealP2 (GGG-ATK-GTY-CCR-AAG-CA) Probe (ACA-SRA-TCT-AYA-TGC-AYC-CTG-C)
	Real-time RT-PCR	Fwd CCRRealP1 (TCT-TYG-CHG-ATG-AYT-CHT-TYC) Rev CCRRealP2 (GGG-ATK-GTY-CCR-AAG-CA) Probe (ACA-SRA-TCT-AYA-TGC-AYC-CTG-C)
	Nested RT-PCR	Fwd CrCon1+ (RWA-AYG-GRC-TTR-TGG-AYA-CYT-TCA-C) Rev CrCon1- (TRG-CAA-GRC-CKG-TWG-CRA-CWA-GWG-C) Rev Fwd CriCon2+ (ART-GGA-GRA-ARG-AYA-TWG-GYT-TYC-G) Nested Rev CriCon2- (CYT-TGA-YRA-AYT-CYC-TRC-ACC-ABT-C)
All	Real-time RT-PCR	Fwd CCHF-SF2 (GGA-VTG-GTG-VAG-GGA-RTT-TG) Rev CCHF-SR2 (CAD-GGT-GGR-TTG-AAR-GC) Probe CCHF-N2 (CAA-RGG-CAA-RTA-CAT-MAT)
	RT-PCR	Fwd CCS (ATG-CAG-GAA-CCA-TTA-ART-CTT-GGG-A) Rev 1 CCAS1 (CTA-ATC-ATA-TCT-GAC-AAC-ATT-TC) Rev 2 CCAS2 (CTA-ATC-ATG-TCT-GAC-AGC-ATC-TC)
	Real-time RT-PCR	Fwd CC1a_for (GTG-CCA-CTG-ATG-ATG-CAC-AAA-AGG-ATT-CCA-TCT) Rev CC1a_rev (GTG-CCA-CTG-ATG-ATG-CAC-AAA-AGG-ATT-CCA-TCT) Probe CCHF-01 (CAA-CAG-GCT-GCT-CTC-AAG-TGG-AG)

178 (Data and table modified from Gruber *et al.* 2019)

179 2. Serological tests

180 Virus neutralisation assays, generally considered to be highly specific, are rarely used for CCHFV diagnosis.
181 Members of the *Orthonairovirus* genus generally induce a weaker neutralising antibody response than
182 members of other genera in the family *Nairoviridae*. Another drawback is the necessity to perform this assay
183 in high biosafety containment because it uses live virus (Burt *et al.*, 1994; Rodriguez *et al.*, 1997).

184 Currently, there are only a few CCHFV commercial kits for IgM or IgG by ELISA or immunofluorescence (IFA).
185 These are all designed for the human diagnostic market. However, it is possible to adapt these commercial
186 ELISAs and IFAs for serological testing in animals. In addition, some in-house ELISAs have been published
187 for the detection of CCHFV-specific antibodies in animals.

188 Diagnostic performance for humans have been compared between the methods using sensitivity, specificity,
189 concordance and degree of agreement with particular focus on the phase of the infection (Emmerich *et al.*,
190 2021). Available serological test systems detect anti-CCHFV IgM and IgG antibodies accurately, but their
191 diagnostic performance varies with respect to the phase of the infection. In the early and convalescent phases
192 of infection, the sensitivity for detecting specific IgG antibodies differed for the ELISA. Both test systems based
193 on immunofluorescence showed an identical sensitivity for detection of anti-CCHFV IgM antibodies in acute
194 and convalescent phases of infection.

195 IgM antibodies in livestock (sheep, goat and cattle) can be detected by using an IgM-capture ELISA. IgG
196 antibodies can be detected by an IgG-sandwich or indirect ELISA, and total antibodies can be detected by
197 competition ELISA. The benefit of competitive ELISA is the capacity to investigate different animal species,
198 because they are host species independent. Commercial kits for the detection of CCHFV-specific antibodies
199 or the detection of viral antigen are available. The limiting factor for the replication of these protocols in other
200 laboratories is the availability of antigens and (where relevant) specified monoclonal antibodies. Most of the

201 tests described for livestock and wild animals have not undergone a formal validation process (Mertens *et al.*,
202 2013). One of the biggest challenges for such validation studies is the availability of an adequate number of
203 positive well characterised control samples.

204 For information on the availability of reference reagents for use in veterinary diagnostic laboratories, contact
205 the WOAHC Collaborating Centres for Zoonoses in Europe and in Asia-Pacific.

206 C. REQUIREMENTS FOR VACCINES

207 There is no vaccine available for animals.

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317 **NB:** At the time of publication (2023) there was no WOAHO Reference Laboratory for Crimean–Congo
 318 haemorrhagic fever (please consult the WOAHO Web site:
 319 <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>).

320 **NB:** FIRST ADOPTED IN 2014. MOST RECENT UPDATES ADOPTED 2023.

Annexe 9. Chapter 3.3.6. 'Avian tuberculosis'

CHAPTER 3.3.6.

AVIAN TUBERCULOSIS

SUMMARY

Description of the disease: Avian tuberculosis, or avian mycobacteriosis, is ~~an important a significant~~ disease that affects companion, captive exotic, wild, and domestic birds and mammals. The disease is most often caused by Mycobacterium avium subsp. avium (M. a. avium), a member of the M. avium complex. However, more than ten other mycobacterial species have been reported to infect birds. The most significant cause of poultry disease is M. a. avium.

Clinical signs of the disease vary depending on the organs involved. The classical presentation is characterised by chronic and progressive wasting and weakness. ~~Diarrhoea is common and joint swelling are standard features in infected flocks~~. Some birds may show respiratory signs, and occasionally, sudden death occurs. Some birds may develop granulomatous ocular lesions.

Mycobacterium tuberculosis, the agent that most commonly causes human tuberculosis (gene IS61101) is ~~less commonly rarely~~ the cause of infection in birds, and it is often ~~as a the~~ result of transmission from pet bird owners or caretakers of captive birds.

Members of M. avium complex: M. a. avium (serotypes 1–3; containing gene segments insertion sequences IS901 and IS1245), M. avium subsp. hominissuis (serotypes 4–6, 8–11, and 21; lacking gene segment IS901 and containing segment IS1245) and M. intracellulare (serotypes 7, 12–20, and 22–28; lacking both IS901 and IS1245) can also infect an extensive range of mammals such as swine, cattle, deer, sheep, goats, horses, cats, dogs, and exotic species. In humans, all members of the M. avium complex and M. genavense are capable of inducing can induce a progressive disease that is refractory to treatment, ~~mostly—mainly~~ in immunocompromised patients.

~~All manipulations involving~~ Due to the contagious nature of this group of organisms, handling of open live cultures or of material from infected birds must only be carried out with after an appropriate biorisk management risk assessment and the implementation of biosafety measures designed to avoid infection.

Diagnosis of avian tuberculosis in birds depends on the demonstration of ~~the above mentioned a~~ mycobacterial species in live or dead birds or the detection of an immune response, cellular or humoral, culture examination, or gene segments ~~IS6110, IS901 and IS1245~~ by polymerase chain reaction (PCR) in the excretions or secretions of live birds.

Detection of the agent: Where clinical signs of avian tuberculosis are seen in the flock, or typical tuberculous lesions are present in birds at necropsy, the demonstration of acid-fast bacilli in smears or sections made from affected organs is sufficient for a quick positive diagnosis. If acid-fast bacilli are not found but typical tuberculous signs or lesions are present in the birds, a culture of the organism or PCR must be attempted. PCR could also be carried out directly on tissue samples. Any acid-fast organism isolated should be identified by nucleic-acid-based tests or chromatographical (e.g. high-performance liquid chromatography [HPLC]) criteria. Serotyping of isolates of M. avium complex members or PCR for 16S rRNA gene followed by sequencing, or the presence of an amplicon for the insertion sequences IS6110, IS901, and IS1245 could can also be performed. Matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS) is a valuable tool as well for isolates following culture.

43 **Tuberculin test and serological tests:** These tests are ~~normally typically~~ used to determine the
44 disease prevalence of disease in a flock or to detect infected birds. When used to detect the
45 presence of avian tuberculosis in a flock, they should be supported by the necropsy of any birds
46 that give positive reactions.

47 In domestic fowl, the tuberculin test in the wattle is the test of choice. This test is less useful in
48 other species of bird. ~~A better test, especially in waterfowl, is~~ The whole blood stained-antigen
49 agglutination test is better, especially in waterfowl. It is more reliable and ~~has the advantage that~~
50 it will can give a result within a few minutes while the bird is still being held.

51 **Requirements for vaccines and diagnostic biologicals:** No vaccines are available for use in
52 birds. Avian tuberculin purified protein derivative (~~PPD~~) is the standard preparation for use in the
53 tuberculin test of domestic poultry. Avian PPD is also used as a component in the comparative
54 intradermal tuberculin test in cattle (see Chapter 3.1.13 Mammalian tuberculosis [infection with
55 *Mycobacterium tuberculosis* complex]).

56 A. INTRODUCTION

57 Several mycobacterial species can be involved in the aetiology of avian tuberculosis ~~and, also known as~~ avian
58 mycobacteriosis. Avian tuberculosis is most commonly ~~produced~~ caused by infection with *Mycobacterium*
59 *avium* subsp. *avium* (serotypes 1, 2, and 3: containing specific gene segment IS901 and nonspecific segment
60 IS1245) and less frequently by *M. genavense* (Guerrero *et al.*, 1995; Pavlik *et al.*, 2000; Salamatian *et al.*,
61 2020; Sattar *et al.*, 2021; Tell *et al.*, 2001). Avian mycobacteriosis is also caused by other two members of the
62 *M. avium* complex: *M. avium* subsp. *hominissuis* (serotypes 4–6, 8–11, and 21: lacking gene segment IS901
63 and containing segment IS1245 and mainly infecting humans and pigs) and *M. intracellulare* (serotypes 7, 12–
64 20, and 22–28: lacking both gene segments IS901 and IS1245) and by ~~*M. intracellulare*, *M. scrofulaceum*,~~
65 ~~*M. fortuitum*,~~ and other potentially pathogenic mycobacterial species including *M. scrofulaceum* and
66 *M. fortuitum*. Under some circumstances, an extensive range of mammalian species, such as swine, cattle,
67 deer, sheep, goats, horses, cats, dogs, and exotic animals, can be infected by these mycobacterium species
68 (Dvorska *et al.*, 2004; Kunze *et al.*, 1992; Mijs *et al.*, 2002; Shitaye *et al.*, 2009; Tell *et al.*, 2001; Thorel *et al.*,
69 1997; 2001). *Mycobacterium tuberculosis* and *M. bovis* are ~~less common as causal~~ rarely the causative agents
70 of tuberculosis in birds (Hoop, 2002; Lanteri *et al.*, 2011; Peters *et al.*, 2007; Schmidt *et al.*, 2022; Tell *et al.*,
71 2001).

72 *Mycobacterium avium* species with standing in nomenclature as of 2023¹ (Arahal *et al.*, 2023) consists of ~~four~~
73 three subspecies: *M. avium* subsp. *avium*, ~~*M. avium* subsp. *hominissuis*,~~ *M. avium* subsp. *silvaticum*, and
74 *M. avium* subsp. *paratuberculosis* (Mijs *et al.*, 2002; Thorel *et al.*, 1990). The latter is the causal agent of
75 Johne's disease, or paratuberculosis, in ruminants and other mammalian species (see Chapter 3.1.16
76 *Paratuberculosis* [Johne's disease]). *Mycobacterium a. silvaticum*, which like *M. avium* subsp.
77 *paratuberculosis* grows *in-vitro* only on media with Mycobactin, which can cause avian tuberculosis in wood
78 pigeons (Thorel *et al.*, 1990). With the widespread use of whole genome sequencing (WGS) and
79 bioinformatics, some studies have investigated the classification of species belonging to the genus
80 *Mycobacterium* and have proposed that *M. avium* comprises three subspecies *M. avium* subsp. *avium*, *M.*
81 *avium* subsp. *paratuberculosis*, and *M. avium* subsp. *lepraemurium*. Further subdividing *M. avium* subsp.
82 *avium* into three variants *M. avium* subsp. *avium* var. *avium*, *M. avium* subsp. *avium* var. *silvaticum*, and
83 *M. avium* subsp. *avium* var. *hominissuis* (Riojas *et al.*, 2021; Tortoli *et al.*, 2019).

84 All *M. a. avium* isolates from birds and mammals, including humans, have a multiple repetitive sequence IS901
85 in their genome and produce a characteristic three band pattern in IS1245 restriction fragment length
86 polymorphism (RFLP) as described and standardised previously (Dvorska *et al.*, 2003; Ritacco *et al.*, 1998).
87 This repetitive sequence is also present in *M. a. silvaticum* and RFLP analysis can help with identification.
88 IS901 has only been detected in *M. avium* strains with serotypes 1, 2 and 3 (Pavlik *et al.*, 2000; Ritacco *et al.*,
89 1998) that are apparently more pathogenic to birds than other serotypes (Tell *et al.*, 2001). On the basis of
90 genetic and phenotypic differences it has recently been proposed to differentiate *M. a. avium* into two
91 subspecies based on the target organism: *M. a. hominissuis* for human and porcine isolates and *M. a. avium*
92 for bird type isolates (Mijs *et al.*, 2002). *Mycobacterium a. hominissuis* has polymorphic multiband IS1245
93 RFLP patterns and is able to grow between 24 and 45°C (Mijs *et al.*, 2002; Van Soolingen *et al.*, 1998). It is

¹ <https://psn.dsmz.de/species/mycobacterium-avium>

94 worth noting that the typical features of bird isolates, the three-band pattern in IS1245 RFLP and presence of
95 IS901, have also been found in cervine and bovine isolates of *M. a. avium*.

96 Avian tuberculosis in birds is most prevalent in gallinaceous poultry and in wild birds raised in captivity. Turkeys
97 are quite susceptible, but ducks, geese, and other water birds are comparatively resistant. The practices of
98 allowing poultry to roam at large on the farm (free range) and of keeping the breeders for several years are
99 conducive to the spread of the causal agent of avian tuberculosis among them. Infected individuals and
100 contaminated environments (water and soil) are the main primary sources of infection. The above-mentioned
101 mycobacterial species causing avian tuberculosis can survive for several months in the environment (Dvorska
102 *et al.*, 2007; Kazda *et al.*, 2009; Shitaye *et al.*, 2008; Tell *et al.*, 2001).

103 In most cases, infected birds usually show no clinical signs but they may eventually become lethargic and
104 emaciated. Many affected birds show diarrhoea and swollen joints, and comb and wattles may regress and
105 become pale. Affected birds, especially gallinaceous poultry, are usually older than 1 year. Some show
106 respiratory signs and, including sudden death may occur, dyspnoea is less common, and granulomatous
107 ocular lesions (Pocknell *et al.*, 1996) as well as and skin lesions have been reported. Under intensive
108 husbandry conditions, sudden death may occur, often associated with severe lesions in the liver; such lesions
109 are easily observed at post-mortem examination (Salamatian *et al.*, 2020; Tell *et al.*, 2001).

110 The primary lesions of avian tuberculosis in birds-poultry (chickens and turkeys) are nearly always in the
111 intestinal tract. Such lesions take the form of deep ulcers filled with caseous material containing many
112 mycobacterial cells, and these are discharged into the lumen and appear in the faeces. Before the intestinal
113 tract is opened, the ulcerated areas appear as tumour-like masses attached to the gut wall, but still, when the
114 intestine is opened, the true nature of the mass becomes evident. Typical caseous lesions are nearly always
115 found in the liver and spleen, and; these organs are usually are greatly enlarged because of the formation of
116 new tuberculous tissue. The lungs and other tissues are ordinarily free from lesions even in advanced cases
117 (Salamatian *et al.*, 2020; Tell *et al.*, 2001; Thorel *et al.*, 1997).

118 Among domestic animals (mammals), domestic pigs (*Sus scrofa f. domesticus*) are the most susceptible to
119 avian tuberculosis. Usually, no clinical manifestations are observed in such animals. Avian tuberculosis is
120 suspected when tuberculous lesions are found in the head and mesenteric lymph nodes on meat inspection
121 after slaughter. Findings of tuberculous lesions that involve other organs (liver, spleen, lungs, etc.) are rare,
122 usually occurring at the advanced stage of the disease. *Mycobacterium a. avium* accounted for up to 35% of
123 the *Mycobacteria* isolated from such tuberculous lesions (Dvorska *et al.*, 1999; Pavlik *et al.*, 2003, 2005;
124 Shitaye *et al.*, 2006). Unlike the other species mentioned previously, cattle are highly resistant to the causative
125 agent of avian tuberculosis, and tuberculous lesions are detected in head lymph nodes, or occasionally in liver
126 lymph nodes, only on meat inspection. *Mycobacterium a. avium* can be successfully isolated from tuberculous
127 lesions in mesenteric lymph nodes from juvenile cattle: the isolation rate from cattle under 2 years of age was
128 34.4% in contrast to 13.0% from cattle over 2 years of age (Dvorska *et al.*, 2004).

129 Pet and wild birds with avian mycobacteriosis have clinical presentations often exacerbated by age and viral
130 and fungal co-infections (Schmidt *et al.*, 2022; Schmitz *et al.*, 2018b). The presence of nonspecific clinical
131 signs and the absence of gross finds during necropsy in psittacine and passeriform birds may confound
132 diagnosis. Furthermore, differences in body condition and gross pathology are observed, where psittacines
133 have more severe lesions than passeriform birds. These differences could also be attributed to the fact that
134 they are often more likely infected with *M. genavense* than *M. avium* (Schmitz *et al.*, 2018a). The advent of
135 more affordable WGS has allowed the study of *M. avium* and *M. genavense* and their epidemiology in a large
136 captive population of birds belonging to multiple taxa for over 22 years. In this large bird population, 68% of all
137 birds at necropsy had isolates that were infected with *M. avium* or *M. genavense*. The WGS study of these
138 mycobacterium isolates demonstrated strong evidence of disease clustering among those birds infected with
139 *M. avium* but not among those harbouring *M. genavense* (Witte *et al.*, 2021). This works sheds light on the
140 epidemiology of mycobacterium among captive birds, and future studies are necessary to understand these
141 pathogens' epidemiology better and to help identify its reservoirs.

142 It is essential to bear in mind that all members of *M. avium*-complex and *M. genavense* are capable of giving
143 rise to a progressive disease in humans that is refractory to treatment, especially in immunocompromised
144 individuals (Narsana *et al.*, 2023; Pavlik *et al.*, 2000; Tell *et al.*, 2001). Members of *Mycobacterium avium*
145 complex are classed in Risk Group 2 for human infection and should be handled with appropriate measures
146 All *Mycobacterium* species can cause infection in people (Cowman *et al.*, 2019). Caution should be exercised
147 by those working with birds in environments infected with *Mycobacterium*, especially those

148 immunosuppressed. All laboratory manipulations with live cultures or potentially infected/contaminated
 149 material must be performed at an appropriate biosafety and containment level determined by conducting a
 150 thorough risk assessment as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing*
 151 *biological risk in the veterinary laboratory and animal facilities*. Biocontainment measures should be
 152 determined by risk analysis as described in Chapter 1.1.4. The CDC's online Manual for Biosafety in
 153 Microbiological and Biomedical Laboratories is also a good reference².

154 B. DIAGNOSTIC TECHNIQUES

155 **Table 1.** Test methods available for the diagnosis of avian tuberculosis 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
Detection and identification of the agent^(a)						
Ziehl–Neelsen staining	–	–	–	++	–	–
Culture	–	–	–	++	–	–
Haemagglutination (stained antigen)	+	+++	+	–	++	–
PCR	+++	±	++	+++	±	–
Detection of immune response						
Haemagglutination (stained antigen)	±	+++	±	≡	++	≡
Tuberculin test	++	+++	+	–	++	–

156 Key: +++ = recommended for this purpose; ++ recommended but has limitations;
 157 + = suitable in very limited circumstances; – = not appropriate for this purpose.

158 PCR = polymerase chain reaction.

159 ^(a)A combination of agent identification methods applied to the same clinical sample is recommended.

160 1. Identification of the agent

161 If there is a characteristic history of avian tuberculosis in a flock and typical lesions are found in birds at post-
 162 mortem, the detection of acid-fast bacilli (AFB) in smears or sections from affected organs, stained by the
 163 Ziehl–Neelsen method usually is normally sufficient to establish a diagnosis. Confirmation of *M. avium*
 164 subspecies should be carried out by PCR or other molecular techniques (Kaevska *et al.*, 2010; Slana *et al.*,
 165 2010). Occasionally a case will occur, presumably as a result of due to large infecting doses giving rise to
 166 acute overwhelming disease, in which affected organs, most obviously the liver, have a 'morocco leather'
 167 appearance with fine greyish or yellowish mottling. In such cases AFB may not be found in such cases, but
 168 careful inspection will reveal parallel bundles of brownish refractile bacilli. Prolongation of the hot carbol-fuchsin
 169 stage of Ziehl–Neelsen staining to 10 minutes will usually reveal that these are indeed AFB, with unusually
 170 high resistance to penetration of the stain. Recently, DNA probes and polymerase chain reaction (PCR), and
 171 WGS techniques have been used to identify the agent at the species and subspecies level specifically. Matrix-
 172 assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS) is a valuable tool as
 173 well (Fernández-Esgueva *et al.*, 2021). Traditionally, *M. a. avium* is separated from common nonchromogenic
 174 slow-growing organisms by their ability to grow at 42°C (*M. a. avium*). The method has limited value, as other

² https://www.cdc.gov/labs/pdf/SF__19_308133-A_BMBL6_00-BOOK-WEB-final-3.pdf

175 species are able to grow at 42°C. *Mycobacterium genavense* is particularly fastidious and has special-unique
176 requirements for growth and identification (Shitaye *et al.*, 2010).

177 1.1. Culture

178 If there is a characteristic flock history and suggestive lesions are found at necropsy, but no AFB are
179 seen in smears or sections, an attempt must be made to isolate the causative organism from the
180 necropsy material. The liver or spleen is usually the best organ to use, but if the carcass is
181 decomposed, bone marrow may prove more satisfactory as it could be less contaminated. As with
182 the culture of *M. bovis*, non-sterile specimens need to be processed with detergent, alkali, or acid to
183 eliminate rapidly growing microorganisms before culture (see Chapter 3.1.13 *Mammalian*
184 *tuberculosis [infection with Mycobacterium tuberculosis complex]*). *Mycobacterium a. avium* grows
185 best on media such as Lowenstein-Jensen, Herrold's medium, Middlebrook 7H10 and, 7H11, or
186 Coletsos, with 1% sodium pyruvate added. It may occasionally be is necessary to incorporate
187 mycobactin J, as it is used for the isolation of to isolate *M. a. paratuberculosis genavense* and
188 *M. a. silvaticum*. Growth may be confined to the edge of the condensation water. Cultures should be
189 incubated for at least 8-12 weeks, less if using liquid media. Typically, *M. a. avium* produces 'smooth'
190 colonies within 2-4 weeks; rough variants do occur. Shorter incubation times can be achieved using
191 the liquid culture BACTEC system or the automated fluorescent MGIT 960 culture system.
192 *Mycobacterium a. avium* can also be detected in massively-infected tissue by a conventional PCR,
193 which also allows acceleration of the accelerates pathogen detection and identification (Moravkova
194 *et al.*, 2008). Currently, Direct detection and quantification of *M. a. avium* using IS901 quantitative
195 real-time PCR can be considered as the best fast and inexpensive method (despite its rather high
196 cost per test) (Kaevska *et al.*, 2010; Slana *et al.*, 2010).

197 For *M. genavense*, the optimal medium is liquid media supplemented with Mycobactin J (an iron
198 chelator) and then plated onto a solid medium is such as Middlebrook 7H11 medium acidified to pH 6
199 and supplemented with blood and charcoal (Realini et al., 1999). The incubation period at 37°C with
200 5-7% CO₂ should be extended for at least 6 months 42 days. If samples are directly plated onto solid
201 media, plates should be held for at least 12 weeks. Bacterial growth should be prepared in a smear
202 and stained using an acid-fast stain. All acid-fast organisms should be identified using MALDI-TOF
203 (matrix assisted laser desorption ionisation-time of flight [mass spectrometry]) or PCR (Buckwalter
204 et al., 2016; Hall et al., 2003; Shitaye et al., 2010).

205 Typing of mycobacteria to the species and subspecies level requires a specialised laboratory.
206 Conventional biochemical tests for species identification are lengthy and fail to distinguish between
207 *M. avium* and *M. intracellulare*. Thus, a miscellaneous group of mycobacteria that includes both
208 species is usually classified under the denomination of M. avium complex denomination.
209 Seroagglutination, which is based on the sugar residue specificity of surface glycopeptidolipids,
210 allows classification the parsing of *M. avium* complex organisms into 28 serovars (Wolinsky &
211 Schaefer, 1973). More sophisticated typing methods directed at cell-wall-specific targets are
212 currently available, such as enzyme-linked immunosorbent assays with monoclonal antibodies to
213 major serovars, and high-performance liquid chromatography (HPLC), and WGS. Based on DNA-
214 rRNA hybridisation serovars 1 to 6, 8 to 11, and 21 are currently have been ascribed to *M. a. avium*
215 and *M. a. hominissuis*, and serovars 7, 12 to 20, and 25 to *M. intracellulare*. However, no consensus
216 was achieved on other serovars, and some isolates cannot be serotyped (Inderlied *et al.*, 1993). For
217 final species and subspecies identification, the current methods are WGS and bioinformatic analysis
218 of isolates obtained from sick birds. Avian tuberculosis in birds is commonly caused by *M. a. avium*
219 types 1, 2, or 3. If the isolate is not one of these three serotypes, further molecular identification tests
220 (IS901 PCR) must be carried out conducted in a specialised laboratory. However, it should be borne
221 in mind noted that superficial-tuberculous lesions in caged pet-captive birds, especially psittacines,
222 may be caused by *M. tuberculosis*, and IS6110 PCR should be used for precise identification should
223 always be attempted (Hoop, 2002; Lanteri *et al.*, 2011; Peters *et al.*, 2007; Schmidt *et al.*, 2008; Tell
224 *et al.*, 2001).

225 1.2. Nucleic acid recognition methods

226 Specific and reliable genetic tests for speciation are currently have been available (Saito *et al.*, 1990).
227 , including commercial nucleic acid hybridisation probes have become a 'gold standard' reference

228 method for distinction between distinguishing *M. avium*, and *M. intracellulare* cultures, and
229 *M. genavense* can also be distinguished with these tests. A further probe that covers the whole
230 *M. avium* complex was also developed, as genuine *M. avium* complex strains have been described
231 that fail to react with specific *M. avium* and *M. intracellulare* probes (Soini *et al.*, 1996). Nevertheless,
232 identification errors were reported due to the cross-reactivity, which may have serious consequences
233 (van Ingen *et al.*, 2009). Various in-house molecular methods have been reported for the
234 identification of to identify mycobacterial cultures, including MAC-members of the *Mycobacterium*
235 *avium* complex. The following gene segments could be used to identify *Mycobacterium* isolates as
236 *M. avium* in one multiplex PCR reaction: IS900, IS901, IS1245. The isolates of *M. a. avium*/*M. a.*
237 *silvaticum* are IS900-, IS901+, IS1245+, the isolates of *M. a. hominissuis* are IS900-, IS901-
238 IS1245+, and the isolates of *M. a. paratuberculosis* are IS900+, IS901-, IS1245- (Kaevska *et al.*,
239 2010; Moravkova *et al.*, 2008). A multiplex 16S rRNA PCR and sequencing method for differentiating
240 *M. avium* from *M. intracellulare* and *M. tuberculosis* complex has some advantages (Cousins *et al.*,
241 1996). 16S rRNA is currently commercially available. Similarly, many veterinary diagnostic
242 laboratories commonly perform in-house PCR and sequencing (Kirschner *et al.*, 1993) may also be
243 used. Culture-independent in-house molecular tests have been developed for the detection to detect
244 and identification of identify species belonging to the *M. avium* complex directly from samples (Hall
245 *et al.*, 2003; Kaevska *et al.*, 2010). WGS of isolates has recently become the go-to molecular method
246 to identify mycobacterium isolates from birds with great accuracy. It enables, with the use of
247 bioinformatic tools, not only an accurate identification of species and subspecies, but also helps to
248 determine the organism relatedness within a flock or environment (Witte *et al.*, 2021). In recent years,
249 veterinary diagnostic laboratories have extensively adopted real-time PCR methods to detect
250 *M. a. avium* directly from different specimens (faeces, tissues, formalin-fixed tissues, and
251 environmental samples). The technique rapidly detects fastidious and slow-growing microorganisms,
252 such as *M. a. avium* (Tell *et al.*, 2003a; 2003b).

253 Several commercial diagnostic PCR tests for detecting *M. a. avium* are available. Still, users should
254 consider the skill set and equipment necessary to perform such tests. Furthermore, it is important to
255 determine the fitness for the purpose of these tests before implementation. The interpretation of the
256 results of these molecular tests also requires veterinary expertise.

257 *Mycobacterium a. avium*, the causative agent of avian tuberculosis (Thorel *et al.*, 1990), previously
258 designated as *M. avium* species only, is assigned to serotypes 1 to 3 within the *M. avium* complex
259 of 28 serotypes (Wolinsky & Schaefer, 1973). As revealed by molecular biological studies, the
260 detected insertion sequence IS901 (Kunze *et al.*, 1992) is possessed not only by the isolates of the
261 above-named serotypes, but also by isolates, virulent for birds, that could not be typed because
262 agglutination occurred (Pavlik *et al.*, 2000). In epidemiological studies, a standardised IS901 RFLP
263 methods replaced serotyping (Dvorska *et al.*, 2003).

264 **2. Immunological methods**

265 Tests used for export depend on the importing requirement of individual countries. In the main, the tuberculin
266 test or the haemagglutination (stained antigen) test are most frequently used for export testing of poultry.

267 **2.1. Tuberculin test**

268 The tuberculin test is the most widely used test in-for domestic fowl and the only test for which an
269 international standard for the reagent exists. Tuberculin is the standard avian purified protein
270 derivative (PPD). Birds are tested by intradermal inoculation in the wattle with 0.05 ml or 0.1 ml of
271 tuberculin (containing approximately 2000 International Units [IU]), using a very-fine needle of
272 approximately 26 gauge, 10 mm long × 0.5 mm. The test is read after 48 hours and. A positive
273 reaction is any swelling at the site, from a small firm nodule approximately 5 mm in diameter to gross
274 oedema extending into the other wattle and down the neck. With practice, Even very small wattles
275 on immature birds can be inoculated successfully. However, in immature birds the comb may be
276 used in immature birds, although the results are not so as reliable. Tuberculin testing of the wattle in
277 turkeys is much less reliable-consistent than in the domestic fowl-chickens. Inoculation in the wing
278 web has been recommended as being more efficient, but this is still not as good as for domestic fowl
279 in chickens. Other birds may also be tested in the wing web, but results are not generally satisfactory.
280 The bare ornamental skin areas on Muscovy ducks and some species of pheasant species can be

281 used, but reliability dependability is doubtful, and interpretation is difficult. Testing in the foot web of
282 waterfowl has also been described; the test is not very sensitive and is often complicated by
283 infections of the inoculation site.

284 In the common pheasant (*Phasianus colchicus*), the tuberculin test can be performed in either of two
285 ways. In the first, 0.05 ml or 0.1 ml of tuberculin is injected into the skin of the lower eyelid. A positive
286 result is indicated by marked swelling at the injection site after 48 hours. Alternatively, 0.25 ml of
287 tuberculin is injected into the thoracic muscles, and the birds are observed for 6–10 hours. Infected
288 birds will show signs of depression and keep aside from the flock, and there may be cases of sudden
289 death. No clinical signs will be provoked in uninfected birds.

290 **2.2. Stained antigen test**

291 The stained-antigen agglutination test has been used with good results, especially in domestic and
292 ornamental waterfowl. A drop (0.05–0.1 ml) of the antigen is mixed with the same volume of fresh
293 whole blood, obtained by venipuncture, on a white porcelain or enamel tile. The mixture is rocked for
294 2 minutes and examined for agglutination. The agglutination may be coarse, in which case it is
295 obvious, or quite fine, in which case it may be most clearly seen as an accumulation of the malachite-
296 green-stained antigen around the edge of the drop, leaving the centre a normal blood-red colour.
297 This test is especially useful for screening large flocks for immediate culling and therefore has
298 advantages over the tuberculin test for controlling the disease, even in domestic fowl. It has also
299 been claimed that it is more reliable in domestic poultry than the tuberculin test.

300 **2.2.1. Preparation of the antigen**

301 An antigen stained with 1% malachite green is used for the rapid whole blood plate
302 agglutination test (Rozanska, 1965). The strain used to prepare the stained antigen must be
303 smooth and not auto-agglutinate in saline suspension. It must conform to the characteristics
304 of *M. a. avium*, preferably obtained from a culture collection, to guarantee its authenticity.

305 A strain that will detect infection with any serotype is recommended instead of the specific
306 serotype most likely to be encountered (in Europe, serotype 2 for domestic fowl, serotype 1
307 for waterfowl, and birds and swine in the USA). Using a highly specific strain for the serotype
308 is recommended. The specificity of strains can be determined only by testing them as antigens,
309 although, in general, a serotype 2 antigen will always detect serotype 3 infection and vice
310 versa. Serotype 1 strains detect a wide spectrum of infections and frequently detect infections
311 with mycobactin-dependent mycobacteria or *M. a. silvaticum*. There is no reason not to use a
312 culture containing more than one strain of *M. a. avium* if it shows the desired properties of
313 sensitivity and specificity. Consistency of results between batches will be easier using pure
314 cultures.

315 The organism should be grown in a suitable liquid medium, such as Middlebrook 7H9
316 containing 1% sodium pyruvate. Good growth should be obtained in approximately 7 days.
317 The liquid culture is used as a seed for bulk antigen preparation.

318 Antigen for agglutination tests is best obtained on a solid medium, such as Löwenstein–Jensen
319 or 7H11, containing 1% sodium pyruvate instead of glycerol, using Roux flasks or large bottles.
320 Using a solid medium maximizes the chance of detecting contamination, and antigens grown
321 in some liquid media are not agglutinated by specific antibodies. Liquid seed culture should be
322 diluted (based on experience) to give discrete colonies on the solid medium. This will usually
323 give the best yield increasing the chance of detecting contamination. About 10 ml of inoculum
324 will usually allow it to wash over the whole surface and provide sufficient moisture to keep the
325 air in the bottle near 100% humidity.

326 The bottles are incubated at 37°C, and good growth should be obtained in 14–21 days with
327 most strains. The antigen is harvested by adding sterile glass beads and twice the volume of
328 sterile normal saline (containing 0.3% formalin) as was used to inoculate the bottle. The bottle
329 is then shaken gently to wash off all the growth, and the washing is collected into a sterile
330 bottle and re-incubated at 37°C for 7 days. The killed bacilli are washed twice in sterile normal
331 saline with 0.2% formalin by centrifugation and re-suspension. This sequence is safer than the

332 original method in which the washing was carried out before the incubation that kills the
333 organisms. Finally, bacilli are again centrifuged and re-suspended in sterile normal saline
334 containing 0.2% formalin and 0.4% sodium citrate to a concentration of about 10¹⁰ bacteria
335 per ml. This corresponds to a concentration ten times that which matches tube No. 4 on
336 McFarland's scale.

337 Cultures for antigen should be inspected for contamination daily for the first 5 days of
338 incubation. The suspension made from the culture washings is also re-examined
339 microscopically (for likely contaminants such as yeasts) and rechecked by culture to ensure
340 that the formalin has killed the mycobacteria.

341 **2.2.2. Validation of the antigen**

342 Cultures should be checked by Gram staining for contamination by organisms other than
343 mycobacteria.

344 One or more batches for agglutinating antigen must be tested for efficacy in using serum from
345 naturally or artificially infected tuberculous birds by comparison with a standard preparation of
346 known potency. When using animals for research or reagent testing, approval of the
347 procedures and the use of animals by the institution's ethics committee should be sought
348 before any testing occurs. The potency relative to that of the standard preparation must not
349 differ significantly from that declared on the label. Each bottle of antigen must be tested with
350 normal chicken serum (to detect autoagglutination) and *M. a. avium* positive chicken serum of
351 low and high antibody content. This should be done, where possible, alongside a previous
352 batch of stained antigens. Those bottles that give satisfactory agglutination reactions with the
353 antisera can now be pooled and the antigen stained. This is done by adding 3 ml of 1%
354 malachite green solution per 100 ml of suspension. The stained antigen should be checked
355 using whole blood, just as the unstained antigen was tested with serum. The agglutinating
356 antigen should stay in the refrigerator for at least 6 months at 4°C and much longer if frozen
357 at -20°C or below. If a batch has not been used for several weeks, it should be rechecked,
358 especially for autoagglutination.

359 It is critical to perform a safety test of the unwashed antigen by culture and incubation to ensure
360 that all the bacilli are dead.

361 **Note on limitation of use**

362 Neither the tuberculin test with avian tuberculin nor the stained-antigen agglutination test is likely to be of any
363 value in cases of *M. tuberculosis* infection in ~~caged~~pet birds.

364 **C. REQUIREMENTS FOR DIAGNOSTIC BIOLOGICALS**

365 **1. Background**

366 No vaccines are available.

367 Avian tuberculin is a preparation of purified protein derivatives (PPD-A) made from the heat-treated products
368 of growth of *M. a. avium*. It is used by intradermal injection to reveal delayed hypersensitivity ~~as a means of~~
369 ~~identifying to identify~~ birds infected with or sensitised to the same species of ~~tubercle bacillus~~*Mycobacterium*.
370 Importantly it is also used ~~as an aid to~~ differential diagnosis in the comparative intradermal tuberculin test
371 for bovine tuberculosis (see Chapter 3.1.13). An international standard preparation of PPD-A is being
372 developed by WOAHP to replace the former WHO Standard³.

³ PPD of *M. avium* tuberculin, WHO (1955) Technical Report Series, no.96, 11.

373 The general principles as given in Chapter 1.1.8 *Principles of veterinary vaccine production*, should be followed
374 for injectable diagnostic biologicals such as tuberculin. The standards set out here and in chapter 1.1.8 are
375 intended to be general in nature and may be supplemented by national and regional requirements.

376 2. Outline of production and minimum requirements for tuberculin production

377 2.1. Characteristics of the seed

378 2.1.1. Biological characteristics of the master seed

379 Strains of *M. a. avium* used to prepare seed cultures should be purchased from a culture
380 collection and identified as to species by appropriate tests. Several strains are recommended
381 by for this purpose in different countries. For example, in the European Union (EU), for
382 example, are, D4ER and TB56. Reference may also be made to are recommended. The
383 relevant national recommendations should be followed. Globally there are commercial sources
384 for PPD-A.

385 2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

386 Seed cultures should be shown to be free from contaminating organisms and to be capable of
387 producing tuberculin with of sufficient potency. The necessary tests are described below.

388 2.2. Method of manufacture

389 2.2.1. Procedure

390 The seed material is kept as a stock of freeze-dried cultures. If the cultures have been grown
391 on solid media, it will be necessary to adapt the organism to grow as a floating culture. This is
392 most easily accomplished by incorporating a piece of potato in the flasks of liquid medium (e.g.
393 Watson Reid's medium). When the culture has been adapted to a liquid medium, it can be
394 maintained by a passage at 2–4-week intervals (Angus, 1978; Haagsma & Angus, 1995).

395 The organism is cultivated in modified Dorset-Henley's synthetic medium, then killed by
396 heating in flowing steam and filtered to remove cells. The protein in the filtrate is precipitated
397 chemically (ammonium sulphate or trichloroacetic acid [~~TCA~~] are used), washed, and
398 resuspended. An antimicrobial preservative that does not give rise to false-positive reactions,
399 such as phenol (not more than 0.5% [w/v]), may be added. Mercurial derivatives should not
400 be used. Glycerol (not more than 10% [w/v]) or glucose (2.2% [w/v]) may be added as a
401 stabiliser. The product is dispensed aseptically into sterile neutral glass containers, ~~which are~~
402 then sealed to prevent contamination. The product may be freeze-dried.

403 2.2.2. Requirements for ingredients

404 The production culture substrate must be shown to ~~be capable of producing produce~~ a product
405 that conforms to the ~~standards of the European Pharmacopoeia (2000–2024⁴) standards~~ or
406 other international standards such the WHO (WHO, 1987). It must be free from ingredients
407 known to cause toxic or allergic reactions.

408 2.2.3. In-process controls

409 The production flasks, inoculated from suitable seed cultures, are incubated for the appropriate
410 time period. Any flasks showing contamination or grossly abnormal growth should be
411 discarded after autoclaving. As incubation proceeds, the surface growth of many cultures
412 becomes moist and may sink into the medium or to the bottom of the flask. In PPD-A
413 tuberculin, the pH of the dissolved precipitate (the so-called concentrated tuberculin) should
414 be pH 6.6–6.7. The Kjeldahl method determines the protein level (total organic nitrogen) of the
415 PPD-A concentrate ~~is determined by the Kjeldahl method~~. Total nitrogen and trichloroacetic
416 acid precipitable nitrogen are usually compared.

⁴ https://www.edqm.eu/en/d/234640?p_l_back_url=%2Fen%2Fsearch%3Fq%3Dpurified%2Bprotein%2Bderivative

417 **2.2.4. Final product batch tests**

418 i) Sterility

419 Sterility testing is generally performed according to the European Pharmacopoeia (2000
420 2024) or other guidelines (see ~~also~~ Chapter 1.1.9 *Tests for sterility and freedom from*
421 *contamination of biological materials intended for veterinary use*).

422 ii) Identity

423 One or more batches of tuberculin may be tested for specificity together with a standard
424 preparation of bovine tuberculin by comparing the reactions produced in guinea-pigs
425 sensitised with *M. bovis* using a procedure similar to that described in Section C.2.2.4.iv.
426 ~~In guinea-pigs sensitised with *M. bovis*.~~ The potency of the preparation of avian tuberculin
427 must be shown to be not more than 10% of the potency of the standard preparation of
428 bovine tuberculin used in the potency test. The use of animals for this purpose should be
429 reviewed and approved by your institution's ethical committee.

430 iii) Safety

431 Tuberculin PPD-A can be examined for freedom from living mycobacteria using the
432 culture method described previously. This culture method, which does not require the use
433 of animals, is used in many laboratories, and its use is encouraged over the use of
434 animals for this purpose. ~~The following is the previously described method, using~~
435 ~~experimental animals to evaluate the safety of PPD. The use of animals for this purpose~~
436 ~~should be reviewed and approved by the institution's ethics committee. Two guinea-pigs,~~
437 ~~each weighing not less than 250 g and that have not been treated previously treated with~~
438 ~~any material that will interfere with the test, are injected subcutaneously with 0.5 ml of the~~
439 ~~tuberculin under test. No abnormal effects should occur within 7 days.~~

440 Tests on tuberculin for living mycobacteria may be performed either on the tuberculin
441 immediately before it is dispensed into final containers or on samples taken from the final
442 containers themselves. A sample of at least 10 ml must be taken and ~~this must be~~ injected
443 intraperitoneally or subcutaneously into at least two guinea-pigs, dividing the volume to
444 be tested equally between the guinea-pigs. It is desirable to take a larger sample, 50 ml,
445 and to concentrate any residual mycobacteria by centrifugation or membrane filtration.
446 The guinea-pigs are observed for at least 42 days and are examined macroscopically at
447 post-mortem. Any lesions found are examined microscopically and by culture. Each filled
448 container must be inspected before it is labelled, and any showing abnormalities must be
449 discarded.

450 A test for the absence of toxic or irritant properties must be ~~carried out~~ conducted
451 according to the specifications of the European Pharmacopoeia (2000–2024)
452 specifications or the equivalent regulatory documents for each country or region.

453 To test for lack of sensitising effect, three guinea-pigs that have not previously been
454 treated with any material that could interfere with the test are each injected intradermally
455 on ~~each of three~~ occasions with the equivalent of 500 ~~IU~~ International units – one IU is
456 equal to the biological activity 0.02 µg of PPD – of the preparation under test in a 0.1 ml
457 volume. In the USA and Canada, the potency of the tuberculin is expressed as tuberculin
458 unit (TU) rather than IU. One TU is also defined as 0.02 µgs of PPD. Each guinea-pig,
459 together with ~~each of the~~ three control guinea-pigs that have not been injected previously,
460 is injected intradermally 15–21 days after the third injection with the same dose of ~~the~~
461 ~~same~~ tuberculin. The reactions of the two groups of guinea-pigs should not be
462 significantly different when measured 24–28 hours later.

463 iv) Batch potency

464 The potency of avian tuberculin is determined in guinea-pigs sensitised with *M. a. avium*,
465 ~~by comparison~~ compared with a standard preparation calibrated in IU or TU.

466 Use no fewer than nine albino guinea-pigs, each weighing 400–600 g. Sensitise the
467 guinea-pigs by administering ~~to each, by deep intramuscular injection,~~ a suitable dose of
468 inactivated or live *M. a. avium* to each by deep intramuscular injection. The test is
469 performed between 4 and 6 weeks later ~~as follows: Shave. Briefly, have~~ the guinea-pigs'
470 flanks shaved (an area large enough ~~so as~~ to provide space for three-to-four injections
471 on each side). Prepare at least three dilutions of the tuberculin under test and at least
472 three dilutions of the standard preparation in an isotonic buffer solution containing
473 0.0005% (w/v) polysorbate 80 (Tween 80). Choose the dilutions so that the reactions
474 produced have diameters of not less than 8 mm and not more than 25 mm. Allocate the
475 dilutions to the injection sites randomly ~~according to~~ using a Latin square design. The
476 dilutions correspond to 0.001, 0.0002, and 0.00004 mg of protein in a final dose of 0.2 ml,
477 injected intradermally.

478 At 24 hours, the reactions' diameters ~~of the reactions~~ are measured, and the results are
479 calculated using standard statistical methods, taking the diameters to be directly
480 proportional to the logarithms of the concentrations of the tuberculins. The estimated
481 potency must be not less than 75% and not more than 133% of the potency stated on the
482 label. The test is not valid unless the fiducial limits of error ($p = 0.95$) are not less than
483 50% and not more than 200% of the estimated potency. If the batch fails a potency test,
484 the test may be repeated one or more times, provided that the final estimate of potency
485 and ~~of~~ fiducial limits is based on the combined results of all the tests.

486 It is recommended that avian tuberculin should contain the equivalent of at least
487 25,000 IU/ml or approximately 0.5 mg protein per ml, giving a dose for practical use of
488 2500 IU/0.1 ml.

489 3. Requirements for authorisation/registration/licensing

490 3.1. Manufacturing process

491 The manufacturing process should follow the requirements of European Pharmacopoeia (2000-2024)
492 or other international standards.

493 3.2. Safety requirements

494 3.2.1. Target and non-target animal safety

495 Antimicrobial preservatives or other substances that may be added to a tuberculin must have
496 been shown not to impair the safety and effectiveness of the product. The maximum permitted
497 concentrations for phenol is 0.5% (w/v), and for glycerol, it is 10% (v/v). The pH should be
498 between 6.5 and 7.5.

499 3.2.2. Precautions (hazards)

500 Experience ~~both~~ in humans and animals led to the observation that appropriately diluted
501 tuberculin injected intradermally results in a localised reaction at the injection site without
502 generalised manifestations. Even in very sensitive persons, severe, generalised reactions are
503 extremely rare and limited.

504 3.3. Stability

505 During storage, liquid avian tuberculin should be protected from the light and held at a temperature
506 of 5°C ($\pm 3^\circ\text{C}$). Freeze-dried preparations may be stored at higher temperatures (~~but~~ not exceeding
507 25°C) and protected from ~~the~~ light. During use, periods of exposure to higher temperatures or to
508 direct sunlight should be kept at a minimum.

509 ~~Provided the tuberculins are~~ Following accepted practice, tuberculin should be stored at a
510 temperature of between 2°C and 8°C and protected from light; they may be used up to the end of
511 the following periods ~~subsequent to~~ after the last satisfactory potency test: Liquid PPD tuberculins:
512 2 years; lyophilised PPD-A tuberculins: 8 years; HCSM (heat-concentrated synthetic-medium)

513 tuberculins diluted: 2 years. Recent research on the temperature stability of human, bovine, and
514 avian tuberculin solutions has shown that they are stable for a year at 37°C. This should be further
515 explored as these products are used in the field in remote areas of the world where maintaining
516 temperature control is very difficult (Maes et al., 2011).

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680 *

681 * *

682 **NB:** There is currently (2024) no WOA Reference Laboratory for avian tuberculosis
683 (please consult the WOA Web site for the current list:

684 <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3http://www.oie.int/>).

685 **NB:** FIRST ADOPTED IN 1989 AS TUBERCULOSIS IN BIRDS. MOST RECENT UPDATES ADOPTED IN 2014.

Annexe 10. Chapter 3.4.1. 'Bovine anaplasmosis'

SECTION 3.4.

BOVINAE

CHAPTER 3.4.1.

BOVINE ANAPLASMOSIS

SUMMARY

Definition of the disease: Bovine anaplasmosis results from infection with *Anaplasma marginale*. A second species, *A. centrale*, has long been recognised and usually causes benign infections. *Anaplasma marginale* is responsible for almost all outbreaks of clinical disease. *Anaplasma phagocytophilum* and *A. bovis*, which infect cattle, ~~have been recently~~ are also included within the genus but they are not reported to. *Anaplasma phagocytophilum* can cause clinical self-limiting disease in cattle. There are no reports of disease associated with *A. bovis* infection. The organism is classified in the genus *Anaplasma* belonging to the family Anaplasmataceae of the order Rickettsiales.

Description of the disease: Anaemia, jaundice in acute, severe cases and sudden unexpected death are characteristic signs of bovine 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 molecular amplification techniques. The disease is typically transmitted by tick vectors, but mechanical transmission by biting insects or by needle can occur.

Detection ~~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* organisms appear as dense, rounded, intraerythrocytic bodies approximately 0.3–1.0 µm in diameter 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 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 spp.* are available in some countries. *Anaplasma phagocytophilum* can only be observed in infected granulocytes, mainly neutrophils and *A. bovis* can only be observed in infected monocytes 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~~ useful 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

40 is the next most frequently used assay. The complement fixation test (CFT) is no longer
41 considered a reliable test ~~for disease certification of individual animals due to variable~~
42 sensitivity. Cross reactivity between *Anaplasma* spp. can complicate interpretation of
43 serological tests. In general, the C-ELISA has the best specificity, with cross-reactivity
44 described between *A. marginale*, *A. centrale*, *A. phagocytophilum* and *Ehrlichia* spp.
45 Alternatively, an indirect ELISA using the CFT with modifications (I-ELISA) is a reliable test
46 used in many laboratories and can be prepared in-house for routine diagnosis of
47 anaplasmosis. Finally, a displacement double-antigen sandwich ELISA has been
48 developed to differentiate between *A. marginale* and *A. centrale* antibodies.

49 ~~**Nucleic-acid-based tests have been used**~~ are often used in diagnostic laboratories and
50 experimentally, and are capable of detecting the presence of low-level infection in carrier
51 cattle and tick vectors. A nested conventional polymerase chain reaction (PCR) reaction is
52 necessary has been used to identify low-level carriers using conventional polymerase chain
53 reaction (PCR), and although nonspecific amplification can occur. Recently, Real-time PCR
54 assays with have analytical sensitivity equivalent to nested conventional PCR have been
55 described and are preferable in a diagnostic setting to reduce the risk of amplicon
56 contamination.

57 **Requirements for vaccines:** Live vaccines are used in several countries to protect cattle
58 against *A. marginale* infection bovine anaplasmosis. A vaccine consisting of live *A. centrale*
59 is most widely used and gives partial protection against challenge with virulent
60 *A. marginale*. Vaccination with *A. centrale* leads to infection and long-term persistence in
61 many cattle. Vaccinated cattle are typically protected from disease caused by *A. marginale*,
62 but not infection.

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

68 *Anaplasma centrale* vaccine is not entirely safe. A practical recommendation is to restrict
69 its use, as far as possible, to calves, as nonspecific immunity will minimise the risk of some
70 vaccine reactions that may require treatment with tetracycline or imidocarb. Partial
71 immunity develops in 6–8 weeks and lasts for several years after a single vaccination. In
72 countries where *A. centrale* is exotic, it cannot be used as a vaccine against *A. marginale*.

73 A. INTRODUCTION

74 Outbreaks of bovine anaplasmosis are due to infection with *Anaplasma marginale*. *Anaplasma centrale*
75 ~~is capable of producing~~ can produce a moderate degree of anaemia, but clinical outbreaks in the field
76 are extremely rare. ~~New species of *Anaplasma*.~~ Other members of the family Anaplasmataceae that
77 infect cattle include *A. phagocytophilum* and *A. bovis* (Dumler *et al.*, 2001), with a primary reservoir,
78 *Anaplasma phagocytophilum* has a broad host range and causes the diseases human granulocytic
79 anaplasmosis (HGE), equine granulocytic anaplasmosis (EGA), and canine granulocytic anaplasmosis
80 (CGA), in humans, horses, and dogs, respectively (Matei *et al.*, 2019). In northern Europe in rodents, *A.*
81 *phagocytophilum* causes tick-borne fever, primarily affecting lambs. In cattle, *A. phagocytophilum*
82 infections have been reported to infect cattle, but do not cause from many geographical regions,
83 however the association with disease is less commonly reported. Naturally occurring clinical disease as
84 reported in Germany was characterised by fever (39.5–41.7° C), sudden reduction in milk production,
85 lower limb oedema, and stiffness with leukopenia, erythropenia, neutropenia, lymphocytopenia and
86 monocytopenia. The affected animals recovered without antibiotic treatment (Dreher *et al.*, 2005;
87 Hofmann-Lehmann *et al.*, 2004 Silaghi *et al.*, 2018).

88 The most marked clinical signs of bovine anaplasmosis are anaemia and jaundice, the latter occurring
89 in acute severe, cases or late in the disease. Haemoglobinaemia and haemoglobinuria are not present,
90 and this may assist in the differential diagnosis of bovine anaplasmosis from babesiosis, which is often
91 endemic in the same regions. The disease can only be confirmed, however, by identification of the
92 organism in erythrocytes from the affected animal. Caution must be exercised if using nucleic acid

93 techniques alone to diagnose *A. marginale* in anaemic cattle. Persistent, low-level infection can be
94 detected by these techniques and may lead to a misdiagnosis of bovine anaplasmosis. Visualisation of
95 *A. marginale* bodies in erythrocytes is therefore required for confirmation.

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

100 ~~*Anaplasma* species were, though originally regarded described as protozoan parasites, but further~~
101 ~~research showed they had no significant attributes to justify this description. Since the last major~~
102 ~~accepted revision of the are obligate intracellular Gram-negative bacteria. Based on taxonomy~~
103 ~~established in 2001 (Dumler *et al.*, 2001), the Family Anaplasmataceae (Order Rickettsiales) is now~~
104 ~~composed of four-five genera, *Anaplasma*, *Ehrlichia*, *Neorickettsia*, and *Wolbachia*. The genus and~~
105 ~~*Aegyptianella* is retained within the Family Anaplasmataceae as genus *incertae sedis*. The revised~~
106 ~~genus. The genus *Anaplasma* now contains *Anaplasma marginale* as the type species,~~
107 ~~*A. phagocytophilum* the agent of human granulocytic ehrlichiosis (formerly *Ehrlichia phagocytophila* and~~
108 ~~*E. equi*), *A. platys*, and *A. bovis* (formerly *E. bovis*). *Haemobartonella* and *Eperythrozoon* are now~~
109 ~~considered most closely related to the mycoplasmas.~~

110 *Anaplasma* species are transmitted either mechanically or biologically by arthropod vectors. Reviews
111 based on careful study Detection of reported transmission experiments list up pathogen DNA within a
112 tick is insufficient to 19 different ticks as capable of determine the ability of a particular tick species to
113 transmit a pathogen. Studies demonstrating transmission of the pathogen are critical in determining the
114 potential role of a particular tick species in pathogen transmission transmitting *A. marginale* (Kocan *et al.*,
115 2004). These are: *Argas persicus*, *Ornithodoros lahorensis*,. Many studies have demonstrated the
116 transmission ability of *Dermcentor albipictus*, *D. andersoni*, *D. hunteri*, *D. occidentalis*, *D. variabilis*,
117 *Hyalomma excavatum*, *H. rufipes*, *Ixodes ricinus*, *I. scapularis*, and *D. albipictus*. Additionally,
118 transmission by multiple *Rhipicephalus* species is well recognised including *R. annulatus* (formerly
119 *Boophilus annulatus*), *R. bursa*, *R. calcaratus*, *R. decoloratus*, *R. evertsi*, *R. microplus*, *R. sanguineus*
120 and *R. simus*. However, the classification of several ticks in these reports has been questioned. and *R.*
121 *sanguineus*. Other species of *Rhipicephalus* also likely serve as biological vectors of *A. marginale*.
122 *Anaplasma marginale* DNA has been widely reported in *Hyalomma* species, and transmission has been
123 demonstrated with *H. excavatum*. It is likely that multiple *Hyalomma* species also serve as vectors of *A.*
124 *marginale* (Shkap *et al.*, 2009).

125 Intrastadial or transstadial transmission is the usual mode can occur, even in the one-host,
126 *Rhipicephalus* species. Male ticks may be particularly important as vectors, as they can become
127 persistently infected and serve as a reservoir are most likely to move between cattle searching
128 for infection-female ticks. Experimental demonstration of vector competence does not necessarily imply a
129 role in transmission in the field. However, *Rhipicephalus* species are clearly important vectors of
130 anaplasmosis in countries such as Australia and countries in many regions of Africa, and Latin America,
131 and some species of *Dermacentor spp.* are efficient vectors in the United States of America (USA).

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

139 The main-only known biological vectors of *A. centrale* appear to be multihost ticks is *R. simus*, endemic
140 in Africa, including *R. simus*. The. Though multiple transmission studies have been done, there is no
141 evidence that the common cattle tick (*R. microplus*) has not been shown to be can serve as a vector for
142 *A. centrale*. This is of relevance-relevant where *A. centrale* is used as a vaccine in *R. microplus*-infested
143 regions.

144 *Anaplasma marginale* infection has not been reported in humans. Thus, There is no minimal risk of field
145 or laboratory transmission to workers and from laboratories working with *A. marginale* may operate at

146 the lowest biosafety level, equivalent to BSL4. Nevertheless the agent should be handled with
 147 appropriate biosafety and containment procedures as determined by biorisk analysis (see Chapter 1.1.4
 148 Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal
 149 facilities).

150

B. DIAGNOSTIC TECHNIQUES

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

Method	Purpose					
	Population freedom from infection ^(a)	Individual animal freedom from infection prior to movement ^(b)	Contribute to eradication policies ^(c)	Confirmation of clinical cases ^(d)	Prevalence of infection – surveillance ^(e)	Immune status in individual animals or populations (post-vaccination) ^(f)
Microscopic examination	–	± _≡	–	+++	–	–
Detection of the agent^(g)						
PCR	–	++ ±	–	+++	–	–
Detection of immune response						
CAT ^(h)	–	–	–	–	+	+
C-ELISA ^(h)	+++	+++	+++	–	+++	+++
IFAT ^(h)	+	–	–	–	++	++
CFT	–	–	–	–	±	–
ddasELISA	≡	≡	≡	≡	≡	±±

152 Key: +++ = recommended for this purpose; ++ recommended but has limitations;

153 + = suitable in very limited circumstances; – = not appropriate for this purpose.

154 Agent id. = agent identification; CAT = card agglutination test; CFT = complement fixation test;

155 C-ELISA = competitive enzyme-linked immunosorbent assay; ddasELISA = displacement double-antigen, sandwich ELISA;

156 IFAT = indirect fluorescent antibody test; PCR = polymerase chain reaction.

157 ^(a)See Appendix 1 of this chapter for justification table for the scores giving to the tests for this purpose.

158 ^(b)See Appendix 2 of this chapter for justification table for the scores giving to the tests for this purpose.

159 ^(c)See Appendix 3 of this chapter for justification table for the scores giving to the tests for this purpose.

160 ^(d)See Appendix 4 of this chapter for justification table for the scores giving to the tests for this purpose.

161 ^(e)See Appendix 5 of this chapter for justification table for the scores giving to the tests for this purpose.

162 ^(f)See Appendix 6 of this chapter for justification table for the scores giving to the tests for this purpose.

163 ^(g)A combination of agent identification methods applied on the same clinical sample is recommended.

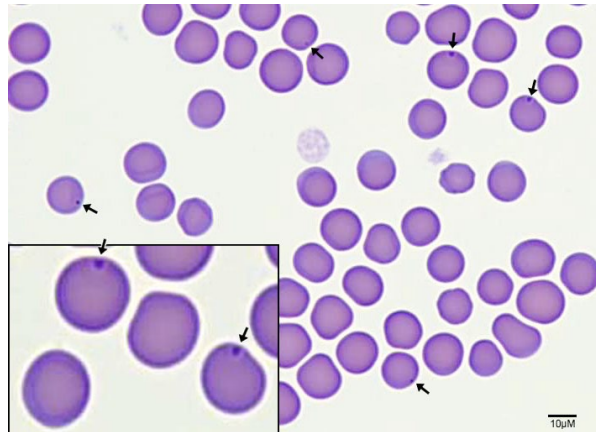
164 ^(h)These tests do not distinguish infected from vaccinated animals.

155 1. Detection of the agent

156 1.1. Microscopic examination

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

175 In contrast to *Babesia bovis*, *A. marginale* ~~does not~~ infected erythrocytes do not accumulate in
176 capillaries, so blood drawn from the jugular or other large vessel is satisfactory. *Anaplasma*
177 *marginale* replicate in the erythrocytes to form small membrane-bound colonies, also termed
178 inclusion bodies or initial-inclusion bodies. Because of the rather indistinctive morphology of
179 *Anaplasma* ~~These~~ initial-inclusion bodies can be visualised on a blood smear, but are small
180 and easily confused with debris or stain precipitate (see Figure 1). Thus it is essential that
181 smears are well prepared ~~and, including ensuring slides are free from foreign matter, as~~
182 ~~specks of debris can confuse diagnosis and stain is recently filtered (Watman #1 filter paper).~~
183 Thick blood films as are used sometimes for the diagnosis of babesiosis are not appropriate
184 for the diagnosis of anaplasmosis, as *Anaplasma* ~~A.~~ *marginale* are difficult to identify once
185 they become dissociated from erythrocytes.



186

187 **Figure 1.** *Anaplasma marginale* initial-inclusion bodies. A Diff-Quick stained blood smear from a
188 bovine experimentally infected with *A. marginale*. Arrows point to the *A. marginale* initial-inclusion
189 bodies.
190 Photo from S. Noh.

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

198 Blood from organs, rather than organ tissues *per se*, is required for smear preparation, as the
199 aim is to microscopically examine intact erythrocytes for the presence of *Anaplasma* ~~A.~~
200 *marginale* colonies. Organ-derived blood smears can be stored satisfactorily at room
201 temperature for several days.

202 Both blood and organ smears can be stained in 10% Giemsa stain for approximately
203 30 minutes after fixation in absolute methanol for 1 minute. After staining, the smears are
204 rinsed three or four times with tap water to remove excess stain and are then air-dried.
205 Conditions for Giemsa staining vary from laboratory to laboratory, but distilled water is not
206 recommended for dilution of Giemsa stock. Water should be pH 7.2–7.4 to attain best
207 resolution with Giemsa stain. Commercial stains that give very rapid staining of *Anaplasma* ~~A.~~
208 *marginale* are available in some countries. Smears ~~are~~ must be examined under oil immersion
209 at a magnification of $\times 700$ – 1000 .

210 *Anaplasma marginale* ~~appear as dense,~~ initial-inclusion bodies are rounded ~~and~~ deeply
211 stained ~~intraerythrocytic bodies, and~~ approximately 0.3–1.0 μm in diameter. Most of these
212 bodies are located on or near the margin of the erythrocyte. This feature distinguishes
213 *A. marginale* from *A. centrale*, as in the latter most of the organisms have a more central
214 location in the erythrocyte. However, particularly at low levels of rickettsaemia, differentiation
215 of these two species in smears can be difficult. Appendages associated with the *Anaplasma*

216 body initial body have been described in some isolates of *A. marginale* (Kreier & Ristic, 1963;
217 Stich et al., 2004).

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

221 The infection becomes visible microscopically 2–6 weeks following transmission. During the
222 course of clinical disease, the rickettsaemia approximately doubles each day for up to about
223 10 days, and then decreases at a similar rate. Severe anaemia may persist for some weeks
224 after the parasites bacteria have become virtually undetectable in blood smears. Following
225 recovery from initial infection, cattle remain latently infected for life.

226 1.2. Polymerase chain reaction

227 Nucleic acid-based tests to detect *A. marginale* infection in carrier infected cattle have been
228 developed although not yet fully validated. The analytical sensitivity of polymerase chain
229 reaction (PCR)-based methods has been estimated at 0.0001% infected erythrocytes, but at
230 this level, only a proportion of carrier cattle would be detected. A nested PCR has been used
231 to identify *A. marginale* carrier cattle with a capability of identifying as few as 30 infected
232 erythrocytes per ml of blood, well below the lowest levels in carriers. However, nested PCR is
233 time consuming as it requires two full PCR reactions, and poses significant quality control and
234 specificity problems for routine use (Torioni De Echaide *et al.*, 1998). Real-time PCR assays
235 are reported to achieve a level of analytical sensitivity equivalent to nested PCR has also been
236 described for identification of *A. marginale* and should be considered instead of the nested
237 PCR (Carelli *et al.*, 2007; Decaro *et al.*, 2008; Reinbold *et al.*, 2010b). Two Advantages of this
238 technique the real-time PCR, which uses a single closed tube for amplification and analysis,
239 are reduced opportunity for risk of amplicon contamination and a semi-quantitative assay
240 result. Equipment and reagents needed for real-time PCR is are expensive, requires
241 preventive maintenance, and may be beyond the capabilities of some laboratories. Real-time
242 PCR assays may target one of several genes (Carelli *et al.*, 2007; Decaro *et al.*, 2008), or 16S
243 rRNA (Reinbold *et al.*, 2010b), and are reported to achieve a level of analytical sensitivity
244 equivalent to nested conventional PCR (Carelli *et al.*, 2007; Decaro *et al.*, 2008; Reinbold *et*
245 *al.*, 2010b).

246 The most widely cited assays for the detection *A. marginale* in individual animals use a probe
247 for increased specificity and are designed to detect *msp1b* (Carelli *et al.*, 2007) or *msp5* (Futse
248 *et al.*, 2003) in genomic DNA extracted from whole blood. The assay based on detection of
249 *msp1b* has been partially validated to detect the pathogen in individual animals and was used
250 to define samples for the validation of a C-ELISA (Carelli *et al.*, 2007; Chung *et al.*, 2014). The
251 analytical test performance of this assay is robust, and exclusivity testing confirmed other
252 bacterial and protozoal tick-borne pathogens of cattle were not detected. The assay, evaluated
253 using 51 blood samples from 18 cattle herds in three regions of southern Italy, had 100%
254 concordance with nested PCR.

255 *Msp1b* is a multigene family. Based on the annotation of the St. Maries strain of *A. marginale*,
256 the designed primers and probe will amplify multiple members of this gene family, including
257 *msp1b-1*, *msp1b-2*, and *msp1-pg3*. This may help increase diagnostic sensitivity, but may
258 pose challenges if quantification of the pathogen is desired. Additionally, some *A. marginale*
259 strains have single nucleotide polymorphisms in *msp1b* within the primer and probe binding
260 regions. Thus, if *msp1b* is used as a diagnostic target, primer and probe design should
261 consider local *A. marginale* strains. *Msp1b* has the advantage as a target in that orthologs of
262 this gene family are absent in the related *A. phagocytophilum* and *Ehrlichia* spp., including *E.*
263 *ruminantium*, thus helping ensure specificity of the test.

264 *Msp5* has also been used as a target to detect *A. marginale* in cattle in field samples and more
265 frequently in experimental samples (Futse *et al.*, 2003). *Msp5* is highly conserved among *A.*
266 *marginale* strains and is a single copy gene, thus providing some advantages as a target for
267 ensuring detection of widely variant strains of *A. marginale*. However, the related *Anaplasma*
268 spp. and *Ehrlichia* spp. all have *msp5* orthologs with 50% identity to an *E. ruminantium* gene

269 (NCBI accession: L07385.1), thus specificity must be determined in laboratory and field
 270 samples. Additionally, little work has been done to validate an *msp5*-based real-time PCR test
 271 for diagnostic purposes.

272 A third primer–probe set is designed to detect *A. marginale* using real-time, reverse
 273 transcriptase PCR. The primers amplify a 16sRNA gene segment from *A. marginale* and *A.*
 274 *phagocytophilum*, while the probe differentiates between the two species (Reinbold *et al.*,
 275 2010b). The analytical performance of this assay is robust. However, the diagnostic sensitivity,
 276 specificity, and of particular importance with 16sRNA sequence-based tests, exclusivity for
 277 other tick-borne pathogens of cattle have not been evaluated. Additionally, this assay is
 278 designed for use following RNA extraction and reverse transcription, which is more laborious
 279 and expensive than DNA extraction. Bacterial RNA is rapidly degraded, and this may ultimately
 280 reduce diagnostic sensitivity of this assay.

281 In regions that use *A. centrale* as a vaccine, it may be useful to differentiate between *A.*
 282 *marginale* and *A. centrale* infected/vaccinated animals. PCR is best suited for this task. The
 283 real-time PCR assay developed by Carelli *et al.* can also be used in a duplex reaction to detect
 284 and differentiate between *A. centrale* and *A. marginale* (Decaro *et al.*, 2008). Primers and
 285 probe have been designed to specifically amplify a region of *A. centrale groEL*, but not *A.*
 286 *marginale groEL*, despite 97% sequence identity between the two genes. The *A. marginale*-
 287 specific primers and probes perform similarly in the single and duplex PCR (Carelli *et al.*,
 288 2007). Using the same 51 field samples from cattle in Italy, the *A. centrale* assay had less
 289 analytical sensitivity compared with nested PCR and discordance in 4 of 51 samples between
 290 an *A. centrale* reverse line blot test and the duplex PCR assay.

291 **Table 2.** Oligonucleotides used in PCR assays to detect *A. marginale* and *A. centrale*

Assay	Reference	Oligonucleotides ^(a)	Sequence 5'–3' ^(b)	Amplicon size (bp)	NCBI accession number
Real-time PCR	Carelli <i>et al.</i> , 2007	<i>Am_msp1b_F</i>	<u>TTG-GCA-AGG-CAG-CAG-CTT</u>	95	M59845
		<i>Am_msp1b_R</i>	<u>TTC-CGC-GAG-CAT-GTG-CAT</u>		
		<i>Am_msp1b_PB</i>	<u>TCG-GTC-TAA-CAT-CTC-CAG-GCT-TTC-AT</u>		
Real-time PCR	Futse <i>et al.</i> , 2003	<i>Am_msp5_F</i>	<u>GCC-AAG-TGA-TGG-TGA-TAT-CGA</u>	151	M93392
		<i>Am_msp5_R</i>	<u>AGA-ATT-AAG-CAT-GTG-ACC-GCT-G</u>		
		<i>Am_msp5_PB</i>	<u>AAC-GTT-CAT-GTA-CCT-CAT-CAA</u>		
Reverse-transcription real-time PCR	Reinbold <i>et al.</i> , 2010	<i>16S rRNA_F</i> ^(c)	<u>CTC-AGA-ACG-AAC-GCT-GG</u>	142	M60313
		<i>16S rRNA_R</i> ^(c)	<u>CAT-TTC-TAG-TGG-CTA-TCC-C</u>		
		<i>Am_16S rRNA_PB</i> ^(d)	<u>CGC-AGC-TTG-CTG-CGT-GTA-TGG-T</u>		
Real-time PCR ^(d)	Decaro <i>et al.</i> , 2008	<i>Ac_groEL_F</i> ^(e, f)	<u>CTA-TAC-ACG-CTT-GCA-TCT-C</u>	77	CP001759.1
		<i>Ac_groEL_R</i> ^(e, f)	<u>CGC-TTT-ATG-ATG-TTG-ATG-C</u>		
		<i>Ac_groEL_PB</i> ^(e, f)	<u>TCA-TCA-TTC-TTC-CCC-TTT-ACC-TCG-T</u>		

292 ^(a)*Am* denotes *A. marginale*, *Ac* denotes *A. centrale*, *Pb* denotes probe sequence.
 293 ^(b)Fluorophores and quenchers not included in probe sequences.
 294 ^(c)Amplifies *A. phagocytophilum* and *A. marginale* 16S rRNA gene.
 295 ^(d)Probe is specific for *A. marginale* 16S rRNA gene.
 296 ^(e)Can be used as a duplex PCR with *msp1b* primers and probe based on Carelli *et al.*, 2007.
 297 ^(f)Primers and probe amplify *A. centrale groEL*.

298 2. Serological tests

299 In general, unless animals have been treated or are at a very early stage of infection (<14 days),
 300 serology using the competitive enzyme-linked immunosorbent assay (C-ELISA), indirect ELISA (I-
 301 ELISA) or card agglutination test (CAT) (see below) may be the preferred methods of identifying infected
 302 animals in most laboratories. *Anaplasma marginale* infections usually persist for the life of the animal.

303 However, except for occasional small recrudescences, *Anaplasma-A. marginale* initial-inclusion bodies
304 cannot readily be detected in blood smears after acute rickettsaemia and, ~~even~~-end-point PCR may not
305 detect the presence of *Anaplasma*-the pathogen in blood samples from asymptomatic carriers. Thus, a
306 number of serological tests have been developed with the aim of detecting persistently infected animals.

307 A feature of the serological diagnosis of anaplasmosis is the highly variable results with regard to both
308 sensitivity and specificity reported for many of the tests from different laboratories. This is due at least
309 in part to inadequate evaluation-validation of the tests using significant numbers of known positive and
310 negative animals. ~~Importantly, the capacity of several assays to detect known infections of long-standing~~
311 ~~duration has been inadequately addressed.~~ An exception is a C-ELISA (see below), which has been
312 was initially validated using true positive and negative animals defined by nested PCR (Torioni De
313 Echaide *et al.*, 1998), ~~and the card agglutination assay, for which relative sensitivity and specificity in~~
314 ~~comparison with the C-ELISA has been evaluated (Molloy *et al.*, 1999).~~ And updated in 2014 (Chung *et*
315 *al.*, 2014). Therefore, while most of the tests described in this section are useful for obtaining broad-
316 based epidemiological data, caution is advised on their use for disease certification. The C-ELISA, I-
317 ELISA and CAT are described in detail below.

318 It should be noted that there is a high degree of cross-reactivity between *A. marginale* and *A. centrale*,
319 as well as cross-reactivity with both *A. phagocytophilum* and *Ehrlichia* spp. in serological tests (Al-
320 Adhami *et al.*, 2011; Dreher *et al.*, 2005). While the infecting species can sometimes be identified using
321 antigens from homologous and heterologous species, equivocal results are obtained on many
322 occasions. Efforts have been made to develop tests that differentiate between naturally acquired
323 immunity to *A. marginale* and vaccine acquired immunity due to immunisation with *A. centrale*
324 *et al.*, 2023; Sarli *et al.*, 2020).

325 2.1. Competitive enzyme-linked immunosorbent assay

326 A C-ELISA using a recombinant antigen termed Major surface protein 5 (MSP5) is an
327 immunodominant protein expressed by *A. marginale*, *A. ovis*, and *A. centrale*. In *A. marginale*
328 the gene is highly conserved making it a useful target across broad geographical regions with
329 high *A. marginale* strain diversity (Knowles *et al.*, 1996; Torioni De Echaide *et al.*, 1998). Thus,
330 a C-ELISA based on recombinantly expressed (rMSP5 and MSP5-) in combination with an
331 MSP5-specific monoclonal antibody (mAb) has proven very sensitive and specific for detection
332 of *Anaplasma*-infected animals (Hofmann-Lehmann *et al.*, 2004; Molloy *et al.*, 1999; Reinbold
333 *et al.*, 2010b; Strik *et al.*, 2007). All *A. marginale* strains tested, along with Additionally, *A. ovis*
334 and *A. centrale*, express the MSP5 antigen and induce infected animals produce antibodies
335 against the immunodominant epitope recognised by the MSP5-specific mAb. A recent report
336 mAb used in the C-ELISA. This C-ELISA was updated in 2014 to improve performance by
337 using glutathione S-transferase (GST) instead of maltose binding protein (MBP) as the tag on
338 the rMSP5 (Chung *et al.*, 2014). This assay no longer requires adsorption to remove the
339 antibodies directed against MBP, thus it is faster and easier than the previous version of the
340 C-ELISA. The diagnostic sensitivity is 100% and the diagnostic specificity is 99.7% using a
341 cut-off of 30% inhibition as determined by receiver operating characteristic (ROC) plot (Chung
342 *et al.*, 2014). For this validation, 385 sera defined as negative were from dairy cattle maintained
343 in tick-free facilities from farms with no clinical history of bovine anaplasmosis. The
344 135 positive sera were from cattle positive for *A. marginale* using nested PCR and serology.

345 One study suggested that antibodies from cattle experimentally infected with
346 *A. phagocytophilum* will test positive in the C-ELISA (Dreher *et al.*, 2005). However, in another
347 study no cross-reactivity could be demonstrated, and the mAb used in the assay did not react
348 with *A. phagocytophilum* MSP5 in direct binding assays (Strik *et al.*, 2007). Cross reactivity
349 has been demonstrated between *A. marginale* and *Ehrlichia* spp. in naturally and
350 experimentally infected cattle (Al-Adhami *et al.*, 2011). Earlier studies had shown that the C-
351 ELISA was 100% specific using 261 known negative sera from a non-endemic region,
352 detecting acutely infected cattle as early as 16 days after experimental tick or blood
353 inoculation, and was demonstrated to detect cattle that have been experimentally infected as
354 long as 6 years previously (Knowles *et al.*, 1996). In detecting persistently infected cattle from
355 an anaplasmosis endemic region that were defined as true positive or negative using a nested
356 PCR procedure, the rMSP5 C-ELISA had a sensitivity of 96% and a specificity of 95% (Torioni

357 ~~De Echaide et al., 1998) *A. marginale* and *Ehrlichia* sp. BOV2010 isolated in Canada, in~~
358 ~~naturally and experimentally infected cattle (Al-Adhami et al, 2011).~~

359 Test results using the rMSP5 C-ELISA are available in less than 2.5-hours. A test kit is
360 available commercially that contains specific instructions. Users should follow the
361 manufacturer's instructions. In general, however, it is conducted as follows.

362 **2.1.1. Kit reagents**

363 A 96-well microtitre plate coated with rMSP5 antigen,
364 A 96-well coated adsorption/transfer plate for serum adsorption to reduce background
365 binding,
366 400×Mab₂peroxidase conjugate,
367 40× wash solution and ready to use conjugate diluting buffer,
368 Ready to use substrate and stop solutions,
369 Positive and negative controls

370 **2.1.2. Test procedure**

- 371 i) ~~Add 70 µl of undiluted serum sample to the coated adsorption/transfer plate and~~
372 ~~incubate at room temperature for 30 minutes.~~
- 373 ii) ~~Transfer 50 µl per well of the adsorbed undiluted serum to the rMSP5-coated plate~~
374 ~~and incubate at room temperature for 60 minutes.~~
- 375 ii) ~~Discard the serum and wash the plate twice using diluted wash solution.~~
- 376 iii) ~~Add 50 µl per well of the 1× diluted MAb₂peroxidase conjugate to the rMSP5-~~
377 ~~coated plate wells, and incubate at room temperature for 20 minutes.~~
- 378 iv) ~~Discard the 1×diluted MAb₂peroxidase conjugate and wash the plate four times~~
379 ~~using diluted wash solution.~~
- 380 v) ~~Add 50 µl per well of the substrate solution, cover the plate with foil, and incubate~~
381 ~~for 20 minutes at room temperature.~~
- 382 vi) ~~Add 50 µl per well of stop solution to the substrate solution already in the wells and~~
383 ~~gently tap the sides of the plate to mix the wells.~~
- 384 vii) ~~Immediately read the plate in the plate reader at 620, 630 or 650 nm.~~

385 **2.1.3. Test validation**

386 The mean average optical density (OD) of the negative control must range from 0.40 to
387 2.10. The average per cent inhibition of the positive control must be ≥30%.

388 **2.1.4. Interpretation of the results**

389 The % inhibition is calculated as follows:

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

390 % inhibition = 100[1 - (Sample OD ÷ Negative Control OD)]

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

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

395 Recently, an improved MSP5 C-ELISA was developed by replacing rMBP-MSP5 with rGST-
396 MSP5 in addition to an improvement in the antigen coating method by using a specific catcher
397 system. The new rMSP5-GST C-ELISA was faster, simpler, had a higher specificity and an
398 improved resolution compared with the rMSP5-MBP C-ELISA with MBP adsorption (Chung *et*
399 *al.*, 2014).

400 2.2. Indirect enzyme-linked immunosorbent assay

401 An I-ELISA was first developed using the CAT antigen, which is a crude *A. marginale* lysate
402 (see below), and it. The test can be implemented where the commercial C-ELISA is not
403 available. Unlike the C-ELISA, most reagents, such as buffers and ready-to dissolve
404 substrates, are available commercially in many countries. Any laboratory can prepare the
405 antigen using local strains of *A. marginale*, though standardised methods have not been
406 developed. I-ELISA uses small amounts of serum and antigen that and the sensitivity and
407 specificity of the test standardised with true positive and negative sera is as good as for the
408 C-ELISA. As it can be prepared in each laboratory, Only the general procedure is described
409 here (Barry *et al.*, 1986). For commercial kits, the manufacturer's instructions should be
410 followed. In the case of in-house I-ELISA. The sensitivity and specificity of the test was 87.3%
411 and 98.4–99.6% respectively, though this varied by laboratory (Nielsen *et al.*, 1996). For
412 general methods, refer to Barry *et al.* (1986). Initial bodies and membranes are obtained as
413 for the complement fixation test (Rogers *et al.*, 1964). This antigen is treated with 0.1% sodium
414 dodecyl sulphate for 30 minutes prior to fixing the antigen to the microtitre plate. For each
415 laboratory, the specific amount of antigen has to must be adjusted/optimised to obtain the best
416 reading and the least expenditure.

417 Alternatively, rMSP5 can be used as the antigen in this test. This eliminates the need for
418 preparation and standardisation of antigen derived from splenectomised, *A. marginale* infected
419 animals (Silva *et al.*, 2006). In a comparison between I-ELISA using the CAT antigen and
420 rMSP with a histidine tag (rMSP5-HIS), these two I-ELISAs performed identically. In this
421 comparison, IFAT was used as the gold standard test (Silva *et al.*, 2006).

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

424 2.2.1. Test reagents

425 A 96-well microtitre plate coated with ~~crude~~ *A. marginale* antigen,
426 PBS/Tween buffer, (PBS 0.1 M, pH 7.2, Tween 20 0.05%),
427 Blocking reagent (e.g. commercial dried skim milk)
428 Tris buffer 0.1 M, MgCl₂, 0.1 M, NaCl, 0.05 M, pH 9.8
429 Substrate *p*-Nitrophenyl phosphate disodium hexahydrate
430 Positive and negative controls.

431 2.2.2. Test procedure (this test is run in triplicate)

- 432 i) Plates can be prepared ahead of time and kept under airtight conditions at –20°C.
- 433 ii) Carefully remove the plastic packaging before using plates, being careful not to
434 touch the bottom of them as this can distort the optical density reading.
- 435 iii) Remove the lid and deposit 200 µl PBST20 solution in each well and incubate at
436 room temperature (RT) for 5 minutes.
- 437 iv) For one plate, dissolve 1.1 g of skim milk (blocking agent) in 22 ml of PBST20.
- 438 v) Remove the plate contents and deposit in each well 200 µl of blocking solution, put
439 the lid on and incubate at 37°C for 60 minutes.
- 440 vi) Wash the plate three times for 5 minutes with PBST20.
- 441 vii) Dilute all serum samples including controls 1/100 in PBST20 solution.

- 442 viii) Remove the contents of the plate and deposit 200 µl of diluted serum in each of
443 the three wells for each dilution, starting with the positive and negative and blank
444 controls.
- 445 ix) Incubate plate at 37°C covered for 60 minutes.
- 446 x) Wash three times as described in **point-subsection** vi.
- 447 xi) Dilute 1/1000 anti-IgG bovine alkaline phosphatase conjugate in PBST20 solution.
448 Add 200 µl of the diluted conjugate per well. Incubate the covered plate at 37°C for
449 60 minutes.
- 450 xii) Remove the lid and wash three times as described in point vi above ~~make three~~
451 ~~washes with PBST20.~~
- 452 xiii) Remove the contents of the plate and deposit 195 µl of 0.075% *p*-Nitrophenyl
453 phosphate disodium hexahydrate in Tris buffer in each well and incubate at 37°C
454 for 60 minutes.
- 455 xiv) The reaction is quantified by a microplate reader spectrophotometer, adjusted to
456 405 nm wavelength. The data are expressed in optical density (OD).

457 2.2.3. Data analysis

458 Analysis of results should take into account the following parameters.

- 459 i) The mean value of the blank wells.
- 460 ii) The mean value of the positive wells with their respective standard deviations.
- 461 iii) The mean value of negative wells with their respective standard deviations.
- 462 iv) The mean value of the blank wells is subtracted from the mean of all the other
463 samples if not automatically subtracted by the ELISA reader.
- 464 v) Control sera are titrated to give optical density values ranging from 0.90 to 1.50 for
465 the positive and, 0.15 to 0.30 for the negative control.

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

468 ~~For purposes of assessing the consistency of the test operator, the error “E” must also be~~
469 ~~estimated; this is calculated by determining the percentage represented by the~~
470 ~~standard deviation of any against their mean serum.~~

471 As with all diagnostic tests, it is important to measure **repeatability-reproducibility**. For
472 more details see Chapter 2.2.4 *Measurement uncertainty*.

473 **2.3. Displacement double-antigen sandwich ELISA to differentiate between *A. marginale*** 474 **and *A. centrale* antibodies**

475 In regions where vaccination with *A. centrale* is used to control bovine anaplasmosis,
476 differentiation between *A. centrale*-vaccinated and *A. marginale*-infected animals may be
477 useful. Because there is often high amino acid identity between *A. marginale* and *A. centrale*
478 surface proteins, identifying unique targets for serological assays for this purpose is difficult.
479 Epitopes from MSP5 (aa28-210, without the transmembrane region) that are not shared
480 between *A. marginale* and *A. centrale* were used to develop a displacement double-antigen
481 sandwich ELISA (ddasELISA) (Bellezze *et al.*, 2023; Sarli *et al.*, 2020). The recombinant
482 MSP5 epitopes from *A. marginale* or *A. centrale* are expressed in *E. coli* with a histidine tag
483 and purified. The ELISA plates are then coated with either the recombinant *A. marginale* MSP5
484 epitope, or the *A. centrale* MSP5 epitope and blocked. Serum is added to the wells and allowed
485 to incubate. Following washing, a combination of biotinylated and non-biotinylated
486 recombinant proteins are added to improve specificity of the reaction (see below for specifics).
487 The protein–biotin binding to the serum antibody is detected with a peroxidase-streptavidin
488 based detection system. The optical density for the *A. marginale* MSP5-coated well (ODAm)
489 and the OD for the *A. centrale* MSP5 (ODAc) coated well for each animal is measured. If the

490 OD for either target is <0.2, the sample is excluded from the analysis. For the remaining
491 samples, the ratio between the OD values (ODAm/ODAc) is calculated. If the ratio is >0.38
492 the sample is considered positive for anti-*A. marginale* antibodies, and a ratio ≤ 0.38 is
493 classified as vaccinated with *A. centrale*.

494 For the detection of *A. marginale* the test has a diagnostic specificity of 98% and a diagnostic
495 sensitivity of 98.9%. For 702 field samples evaluated, 131 (19%) had an OD <0.2 in the
496 ddasELISA and thus were excluded from the analysis. Of those animals, 52% were nested
497 PCR positive for *A. marginale*, 23% were nested PCR positive for *A. centrale*, 4.6% were
498 nested PCR positive for *A. marginale* and *A. centrale*, 20% were nested PCR negative for
499 both, suggesting the ddasELISA may lack sensitivity.

500 Of the 571 ddasELISA positive field samples, the agreement between the ddasELISA and
501 nested PCR was 84% and the kappa coefficient was 0.70 (95% CI: 0.635–0.754), indicating
502 substantial agreement between tests. There was agreement between the ddasELISA and
503 nested PCR for 93% of the *A. marginale* ddasELISA positive samples and 86% of the *A.*
504 *centrale* ddasELISA positive samples. Additionally, 36 nested PCR negative samples tested
505 positive for antibodies against *A. marginale* (n=28) or *A. centrale* (n=8) by ddasELISA. This
506 test could not identify animals with co-infections, meaning animals vaccinated with *A. centrale*
507 that are then infected with *A. marginale*, which is not uncommon.

508 Test results using the ddasELISA are available in 5–6 hours. It is conducted as outlined below,
509 see Bellezze *et al.*, 2023 for more details.

510 **2.3.1. Test reagents**

- 511 i) A 96-well microtitre plate coated with either *A. marginale* or *A. centrale* recombinant
512 protein
- 513 ii) PBS/Tween buffer (PBS (50mM sodium phosphate, 150 mM NaCl, pH 7.2) with
514 0.05% Tween-20)
- 515 iii) Blocking reagent (PBS with 10% commercial dried skim milk)
- 516 iv) Purified recombinant *A. marginale* MSP5 epitopes and *A. centrale* epitopes
- 517 v) Biotinylated recombinant *A. marginale* MSP5 epitopes and *A. centrale* epitopes
- 518 vi) Streptavidin-horse radish peroxidase (HRP) detection system
- 519 vii) Chromogenic substrate (1 mM 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic
520 acid]-diammonium salt in 0.05 M sodium citrate, pH 4.5, 0.0025% V/V H₂O₂ (100
521 µl/well).
- 522 viii) ELISA plate reader (405 nm reading)
- 523 ix) Positive and negative control sera for *A. marginale* and *A. centrale*

524 **2.3.2. Test procedure**

- 525 i) Plates are coated overnight.
- 526 ii) Block with blocking buffer for 1 hour at room temperature and wash three times
527 with PBS/Tween buffer.
- 528 iii) Add undiluted serum 100 µl/well and incubate at 25°C for 1 hour at 100 rpm.
- 529 iv) Wash three times with PBS/Tween buffer.
- 530 v) Add 100 µl of *A. marginale* MSP5-biotin (1 µg/ml) plus *A. centrale* MSP5 (10 µg/ml)
531 to *A. marginale* test wells. Add *A. centrale* MSP5-biotin (1 µg/ml) plus *A. marginale*
532 MSP5 (10 µg/ml) in PBS/Tween buffer + 10% fat-free dried milk to *A. centrale* test
533 wells.
- 534 vi) Incubate at 25°C for 1 hour at 100 rpm and wash the plate five times with
535 PBS/Tween buffer.

- 536 vii) To detect the bound protein–biotin complex, add streptavidin-HRP diluted in 1/500
 537 in PBS/Tween buffer with 10% dried milk for 1 hour at 25°C, 100 rpm.
- 538 vii) Wash five times with PBS/Tween buffer.
- 539 ix) Add chromogenic substrate based on manufacturer's instructions.
- 540 x) The reaction is measured by microplate reader spectrophotometer at 405 nm
 541 wavelength. The data are expressed in optical density (OD).
- 542 xi) $OD_{405nm} < 0.2$ is considered negative.
- 543 xii) Results are expressed as the ratio between antibodies specific for *A. marginale*
 544 MSP5 and for *A. centrale* MSP5 (OD_{Am}/OD_{Ac}). If the ratio is >0.38 the sample is
 545 considered positive for anti-*A. marginale* antibodies, and a ratio ≤ 0.38 is classified
 546 as vaccinated with *A. centrale*.

547 2.4. Card agglutination test

548 ~~The advantages of the CAT are that it is sensitive~~ The sensitivity of the CAT is from 84% to
 549 98% (Gonzalez *et al.*, 1978; Molloy *et al.*, 1999) and the specificity is 98.6% (Molloy *et al.*,
 550 1999). Though sometimes giving variable results, the CAT can be useful under certain
 551 circumstances, as it may be undertaken either in the laboratory or in the field, and it gives a
 552 result within a few minutes. Nonspecific reactions may be a problem, and subjectivity in
 553 interpreting assay reactions can result in variability in test interpretation. In addition, the CAT
 554 antigen, which is a ~~suspension lysate of *A. marginale* particles isolated from erythrocytes~~, can
 555 be difficult to prepare and can vary from batch to batch and laboratory to laboratory. To obtain
 556 the antigen, splenectomised calves are infected by intravenous inoculation with blood
 557 containing ~~Anaplasma~~-*A. marginale*-infected erythrocytes. When the rickettsaemia exceeds
 558 50%, the animal is exsanguinated, the infected erythrocytes are washed, lysed, and the
 559 erythrocyte ghosts and ~~Anaplasma~~ particles-*A. marginale* are pelleted. The pellets are
 560 sonicated, washed, and then resuspended in a stain solution to produce the antigen
 561 suspension.

562 A test procedure that has been slightly modified from that originally described (Amerault &
 563 Roby, 1968; Amerault *et al.*, 1972) is as follows, and is based on controlled conditions in a
 564 laboratory setting:

565 2.4.1. Test procedure

- 566 i) Ensure all test components are at a temperature of 25–26°C before use (this
 567 constant temperature is critical for the test).
- 568 ii) On each circle of the test card (a clear perspex/plastic or glass plate marked with
 569 circles that are 18 mm in diameter), place next to each other, but not touching,
 570 10 μ l of bovine serum factor (BSF), 10 μ l of test serum, and 5 μ l of CAT antigen¹.
 571 Negative and low positive control sera must be tested on each card.
- 572 BSF is serum from a selected animal with high known conglutinin level. If the
 573 conglutinin level is unknown, fresh serum from a healthy animal known to be free
 574 from *Anaplasma* can be used. The BSF must be stored at –70°C in small aliquots,
 575 a fresh aliquot being used each time the tests are performed. The inclusion of BSF
 576 improves the sensitivity of the test.
- 577 iii) Mix well with a glass stirrer. After mixing each test, wipe the stirrer with clean tissue
 578 to prevent cross-contamination.
- 579 iv) Place the test card in a humid chamber and rock at 100–110 rpm for 7 minutes.

¹ The test as conducted in the USA and Mexico uses larger volumes of reagents: antigen (15 μ l), serum (30 μ l), and bovine serum factor (30 μ l), and a 4-minute reaction time (see step iv).

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

583 A latex card agglutination test, a relatively simple and rapid test platform, has been partially
584 validated. This test uses rMSP5-HIS rather than *A. marginale* lysate and does not require BSF.
585 The performance of this test was compared with that of the I-ELISA using rMSP5-HIS as the
586 antigen. The relative sensitivity was 95.2% and relative specificity was 91.86% (Ramos *et al.*,
587 2014).

588 **2.4. Complement fixation test**

589 ~~The complement fixation (CF) test has been used extensively for many years; however, it~~
590 ~~shows variable sensitivity (ranging from 20 to 60%), possibly reflecting differences in~~
591 ~~techniques for antigen production, and poor reproducibility. In addition, it has been~~
592 ~~demonstrated that the CF assay fails to detect a significant proportion of carrier cattle~~
593 ~~(Bradway *et al.*, 2001). It is also uncertain as to whether or not the CF test can identify~~
594 ~~antibodies in acutely infected animals prior to other assays (Coetzee *et al.*, 2007; Molloy *et al.*,~~
595 ~~1999). Therefore, the CF test is no longer recommended as a reliable assay for detecting~~
596 ~~infected animals.~~

597 **2.5. Indirect fluorescent antibody test**

598 Because of the limitations on the number of indirect fluorescent antibody (IFA) tests that can
599 be performed daily by one operator, other serological tests are generally preferred to the IFA
600 test. The IFA test is performed as described for bovine babesiosis in chapter 3.4.2, except that
601 *A. marginale* infected blood is used for the preparation of antigen smears. A serious problem
602 encountered with the test is nonspecific fluorescence. The reported sensitivity is 97.6% and
603 specificity 89.6% (Gonzalez *et al.*, 1978). Antigen made from blood collected as soon as
604 adequate rickettsaemia (5–10%) occurs is most likely to be suitable. Nonspecific fluorescence
605 due to antibodies adhering to infected erythrocytes can be reduced by washing the
606 erythrocytes in an acidic glycine buffer before antigen smears are prepared. Infected
607 erythrocytes are washed twice in 0.1 M glycine buffer (pH 3.0, centrifuged at 1000 *g* for
608 15 minutes at 4°C) and then once in PBS, pH 7.4. Recently published data show that the IFA,
609 like the C-ELISA, can cross react with other members of the *Anaplasmataceae* family, and
610 specifically an *Ehrlichia* spp. identified as BOV2010 (Al-Adhami *et al.*, 2011).

611 **2.6. Complement fixation test**

612 The complement fixation test (CFT) was used extensively for many years; however, it has
613 variable sensitivity (ranging from 20 to 60%), possibly reflecting differences in techniques for
614 antigen production, and poor reproducibility. In addition, the CF assay fails to detect a
615 significant proportion of carrier cattle (Bradway *et al.*, 2001). It is also uncertain as to whether
616 or not the CF test can identify antibodies in acutely infected animals prior to other assays
617 (Coetzee *et al.*, 2007; Molloy *et al.*, 1999). Therefore, the CF test is no longer recommended
618 as a reliable assay for detecting infected animals.

619 **C. REQUIREMENTS FOR VACCINES**

620 **1. Background**

621 Several immunisation methods have been used to protect cattle against anaplasmosis in countries
622 where the disease is endemic, but none is ideal to date (McHardy, 1984). A review of *A. marginale*
623 vaccines and antigens has been published (Kocan *et al.*, 2003–2010; Noh *et al.*, 2012). Use of the less
624 pathogenic *A. centrale*, which gives partial cross-protection against *A. marginale*, is the most widely
625 accepted method, although not used in many countries ~~where the disease is exotic~~, including north
626 America.

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

631 Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary*
632 *vaccine production*. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature
633 and may be supplemented by national and regional requirements.

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

639 2. Outline of production and minimum requirements for conventional vaccines

640 2.1. Characteristics of the seed

641 2.1.1. Biological characteristics

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

649 *Anaplasma centrale* usually causes benign infections, especially if used in calves under
650 9 months of age. Severe reactions following vaccination have been reported when adult
651 cattle are inoculated. The suitability of an isolate of *A. centrale* as a vaccine can be
652 determined by inoculating susceptible cattle, monitoring the subsequent reactions, and
653 then challenging the animals and susceptible controls with a virulent local strain of
654 *A. marginale*. Both safety and efficacy can be judged by monitoring rickettsaemias in
655 stained blood films and the depression of packed cell volumes of inoculated cattle during
656 the vaccination and challenge reaction periods.

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

668 2.1.2. Quality criteria

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

678 enzootic bovine leukosis, mucosal disease, infectious bovine rhinotracheitis, ephemeral
679 fever, Akabane disease, bluetongue, and foot and mouth disease, ~~and rinderpest~~. The
680 testing procedures will depend on the diseases prevalent in the country and the
681 availability of tests but should involve serology of paired sera at the very least and, in
682 some cases, virus isolation, antigen, or DNA/RNA detection (Bock *et al.*, 2004; Pipano,
683 1981; 1997).

684 2.2. Method of manufacture

685 2.2.1. Procedure

686 i) Production of frozen vaccine

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

691 The rickettsaemia of ~~the~~ this donor calf is monitored daily by examining stained
692 films of jugular blood, and the blood is collected for vaccine production when
693 suitable rickettsaemias are reached. A rickettsaemia of 1×10^8 /ml (approximately
694 2% rickettsaemia in jugular blood) is the minimum required for production of
695 vaccine as this is the dose to vaccinate a bovine. If a suitable rickettsaemia is not
696 obtained, passage of the strain by subinoculation of 100–200 ml of blood to a
697 second splenectomised calf may be necessary.

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

703 In the laboratory, the infective blood is mixed in equal volumes with 3 M glycerol in
704 PBS supplemented with 5 mM glucose at 37°C (final concentration of glycerol
705 1.5 M). The mixture is then equilibrated at 37°C for 30 minutes and dispensed into
706 suitable containers (e.g. 5 ml cryovials). The vials are cooled at approximately
707 10°C/minute in the vapour phase of liquid nitrogen and, when frozen, stored in the
708 liquid phase (Bock *et al.*, 2004).

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

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

716 ii) Production of chilled vaccine

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

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

726 **iii) Use of vaccine**
727 In the case of frozen vaccine, vials should be thawed by immersion in water,
728 preheated to 37°C to 40°C, and the contents mixed with suitable diluent to the
729 required dilution. If glycerolised vaccine is prepared, it should be kept cool and
730 used within 8 hours (Bock *et al.*, 2004). If DMSO is used as a cryoprotectant, the
731 prepared vaccine should be kept on ice and used within 15–30 minutes (Pipano,
732 1981). The vaccine is most commonly administered subcutaneously.

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

735 The strain of *A. centrale* used in the vaccine is of reduced virulence, but is not
736 entirely safe. A practical recommendation is, therefore, to limit the use of vaccine
737 to calves, where nonspecific immunity will minimise the risk of vaccine reactions.
738 When older animals have to be vaccinated, there is a risk of severe reactions.
739 These reactions occur infrequently, but valuable breeding stock or pregnant
740 animals obviously warrant close attention, and should be observed daily for
741 3 weeks post-vaccination. Clinically sick animals should be treated with
742 oxytetracycline or imidocarb at dosages recommended by the manufacturers.
743 Protective immunity develops in 6–8 weeks and usually lasts for several years.

744 Anaplasmosis and babesiosis vaccines are often used concurrently, but it is not
745 advisable to use any other vaccines at the same time (Bock *et al.*, 2004).

746 **2.2.2. Requirements for substrates and media**

747 *Anaplasma centrale* ~~cannot~~ can be cultured in ~~vitro~~ *Rhipicephalus appendiculatus* and
748 *Dermacentor variabilis* cells lines, though antigen expression and immunogenicity of the
749 cultured *A. centrale* need to be tested (Bell-Sakyi *et al.*, 2015). No substrates or media
750 other than buffers and diluents are used in vaccine production. DMSO or glycerol should
751 be purchased from reputable companies.

752 **2.2.3. In-process controls**

753 **i) Source and maintenance of vaccine donors**

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

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

764 **ii) Surgery**

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

768 **iii) Screening of vaccine donors before inoculation**

769 As for preparation of seed stabilate, donor calves for vaccine production should be
770 examined for all blood-borne infections prevalent in the vaccine-producing country,
771 including *Babesia*, *Anaplasma*, *Ehrlichia*, *Theileria* and *Trypanosoma*. This can be
772 done by routine examination of stained blood films after splenectomy, and
773 preferably also by serology. Any calves showing evidence of natural infections of
774 any of these agents should be rejected. The absence of other infective agents

775 should also be confirmed. These may include the agents of enzootic bovine
776 leukosis, bovine viral diarrhoea, infectious bovine rhinotracheitis, ephemeral fever,
777 Akabane disease, bluetongue, and foot and mouth disease. The testing
778 procedures will depend on the diseases prevalent in the country and the availability
779 of tests, but should involve serology of paired sera at the very least and, in some
780 cases, virus isolation, antigen, or DNA/RNA detection (Bock *et al.*, 2004; Pipano,
781 1981; 1997).

782 **iv) Monitoring of rickettsaemias following inoculation**

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

786 **v) Collection of blood for vaccine**

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

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

795 **vi) Dispensing of vaccine**

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

801 **2.2.4. Final product batch tests**

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

805 **i) Sterility and purity**

806 Standard tests for sterility are employed for each batch of vaccine and diluent (see
807 Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological*
808 *materials intended for veterinary use*).

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

824 are many publications describing primers, and assay conditions for any particular
825 disease.

826 ii) Safety

827 Vaccine reactions of the cattle inoculated in the test for potency (see Chapter 1.1.8
828 *Principles of veterinary vaccine production*) are monitored by measuring
829 rickettsaemia and depression of packed cell volume. Only batches with
830 pathogenicity levels equal to or lower than a predetermined standard are released
831 for use.

832 iii) Potency

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

839 2.3. Requirements for authorisation

840 2.3.1. Safety

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

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

853 2.3.2. Efficacy requirements

854 ~~Partial but long-lasting immunity results from one inoculation. There is no evidence that~~
855 ~~repeated vaccination will have a boosting effect. Immunisation with live *A. centrale*~~
856 ~~results in long-term infection of the vaccinee, thus repeated vaccination is unnecessary.~~
857 ~~Infection with *A. centrale* does not prevent subsequent infection with *A. marginale*, but~~
858 ~~does at least result in protection from disease (Shkap *et al.*, 2009).~~ The vaccine is used
859 for control of clinical anaplasmosis in endemic areas. It will not provide sterile immunity,
860 and should not be used for eradication of *A. marginale*.

861 2.3.3. Stability

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

864 3. Vaccines based on biotechnology

865 There are no vaccines based on biotechnology available for anaplasmosis.

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1003 nested PCR and a competitive enzyme-linked immunosorbent assay using recombinant major surface
1004 protein 5. *J. Clin. Microbiol.*, **36**, 777–782.

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1007 **NB:** There is a WOAHA Reference Laboratory for anaplasmosis (please consult the WOAHA Web site:
1008 <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>)
1009 Please contact the WOAHA Reference Laboratory for any further information on
1010 diagnostic tests, reagents and vaccines for bovine anaplasmosis

1011 **NB:** FIRST ADOPTED IN 1991. MOST RECENT UPDATES ADOPTED IN 2015.

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Appendix 1: Bovine anaplasmosis
Intended purpose of test: population freedom from infection

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>C-ELISA +++ Bovine</u>	<u>Serum rMSP5-GST</u>	<u>Reference tests were nested PCR and IFAT. Dsp = 99.7% Dse = 100% 30% inhibition as determined by ROC analysis.</u>	<u>1. 358 known non-infected cattle from dairy herds maintained in tick free barns and no clinical history of clinical anaplasmosis. 2. 135 known positive sera as defined by nested PCR. 3. Intra-test comparison with 163 diagnostic samples with possible false positives based on rMSP5-GST C-ELISA. Test positive confirmation done with IFAT.</u>	<u>See reference</u>	<u>1. Updated version with improved specificity. 2. High sensitivity, detects persistently infected animals. 3. Commercially available. 4. Uses a standardised antigen. 5. Target antigen is highly conserved among <i>A. marginale</i> strains, thus detects infection with all strains of <i>A. marginale</i>. 6. Rapid.</u>	<u>1. Does not differentiate between infection with <i>A. marginale</i> and <i>A. centrale</i>. 2. May cross react with anti-<i>Ehrlichia</i> antibodies. 3. May not be readily available in all countries. 4. Requires a microplate absorbance reader. 5. Low percent of false positive results.</u>	<u>Chung <i>et al.</i>, 2014.</u>
<u>IFAT+ Bovine</u>	<u>Serum Glass slides with RBCs infected with <i>A. marginale</i>.</u>	<u>Reference test was blood smear. DSe 97.6% Dsp 89.6%</u>	<u>48 cattle raised in anaplasmosis free region. 82 animals from endemic region.</u>	<u>See reference</u>	<u>1. Antigen is relatively easy to produce and store. 2. Does not require many reagents.</u>	<u>1. Low specificity. 2. Time consuming and labour intensive so not suitable for high throughput. 3. Requires fluorescent microscope and blood smears with high rickettsemia.</u>	<u>Gonzalez <i>et al.</i>, 1978</u>

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Appendix 2: Bovine anaplasmosis
Intended purpose of test: Individual animal freedom from infection prior to movement.

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
PCR ++	Whole blood Various gene targets	Partial validation has been published.	51 cattle from 18 herds in three regions of southern Italy were tested by RLB ¹ for <i>A. marginale</i> , <i>A. centrale</i> , <i>A. bovis</i> , <i>T. buffeli</i> , <i>B. bovis</i> , <i>A. phagocytophilum</i> , and <i>B. bigemina</i> . All cattle except 4 were positive for at least one of these pathogens.	See reference	Good reported concordance between nested PCR and real time PCR. High analytic sensitivity (10 ¹ DNA copies).	Must be performed in a lab equipped to extract DNA and have thermocyclers for real time PCR. Though not determined empirically, it is likely that PCR has less sensitivity than C-ELISA in detection of persistently infected cattle.	Carelli <i>et al.</i> , 2007.
C-ELISA +++ Bovine	Serum rMSP5-GST	Reference tests were nested PCR and IFAT. Dsp = 99.7% Dse = 100% 30% inhibition as determined by ROC analysis.	1. 358 known non-infected cattle from dairy herds maintained in tick free barns and no clinical history of clinical anaplasmosis. 2. 135 known positive sera as defined by nested PCR. 3. Intra-test comparison with 163 diagnostic samples with possible false positives based on rMSP5-GST C-ELISA. Test positive confirmation done with IFAT.	See reference	1. Updated version with improved specificity. 2. High sensitivity, detects persistently infected animals. 3. Commercially available. 4. Uses a standardised antigen. 5. Target antigen is highly conserved among <i>A. marginale</i> strains, thus detects infection with all strains of <i>A. marginale</i> . 6. Rapid.	1. Does not differentiate between infection with <i>A. marginale</i> and <i>A. centrale</i> . 2. May cross react with anti- <i>Ehrlichia</i> antibodies. 3. May not be readily available in all countries. 4. Requires a microplate absorbance reader.	Chung <i>et al.</i> , 2014.

5 ¹RLB is the reverse line blot test.

Appendix 3: Bovine anaplasmosis
Intended purpose of test: contribute to eradication policies

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>C-ELISA +++ Bovine</u>	<u>Serum rMSP5-GST</u>	<u>Reference tests were nested PCR and IFAT. Dsp = 99.7% Dse = 100% 30% inhibition as determined by ROC analysis.</u>	<u>1. 358 known non-infected cattle from dairy herds maintained in tick free barns and no clinical history of clinical anaplasmosis. 2. 135 known positive sera as defined by nested PCR. 3. Intra-test comparison with 163 diagnostic samples with possible false positives based on rMSP5-GST C-ELISA. Test positive confirmation done with IFAT.</u>	<u>See reference</u>	<u>1. Updated version with improved specificity. 2. High sensitivity, detects persistently infected animals. 3. Commercially available. 4. Uses a standardised antigen. 5. Target antigen is highly conserved among <i>A. marginale</i> strains, thus detects infection with all strains of <i>A. marginale</i>. 6. Rapid.</u>	<u>1. Does not differentiate between infection with <i>A. marginale</i> and <i>A. centrale</i>. 2. May cross react with anti-<i>Ehrlichia</i> antibodies. 3. May not be readily available in all countries. 4. Requires a microplate absorbance reader. 5. Low percent of false positive results.</u>	<u>Chung <i>et al.</i>, 2014)</u>

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Appendix 4: Bovine anaplasmosis
Intended purpose of test: confirmation of clinical cases

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Microscopic examination +++</u>	<u>Whole blood</u>	<u>No robust validation has been published.</u>	<u>N/A</u>	<u>N/A</u>	<u>1. Most laboratories have the capacity to make and examine blood smears.</u> <u>2. <i>A. marginale</i> infected erythrocytes readily visible in clinically affected animals.</u>	<u>1. <i>A. marginale</i> colonies are small and can be difficult to differentiate from debris if animal has low rickettsemia.</u> <u>2. Requires experience to identify <i>A. marginale</i> colonies.</u> <u>3. Difficult to differentiate between <i>A. marginale</i> and <i>A. centrale</i>.</u>	
<u>PCR +++</u>	<u>Whole blood</u> <u>Various gene targets</u>	<u>Partial validation has been published.</u>	<u>51 cattle from 18 herds in three regions of southern Italy were tested by RLB¹ for <i>A. marginale</i>, <i>A. centrale</i>, <i>A. bovis</i>, <i>T. buffeli</i>, <i>B. bovis</i>, <i>A. phagocytophilum</i>, and <i>B. bigemina</i>. All cattle except 4 were positive for at least one of these pathogens.</u>	<u>See reference</u>	<u>Good reported concordance between nested PCR and real time PCR. High analytic sensitivity (10¹ DNA copies).</u>	<u>1. Must be performed in a lab equipped to extract DNA and have thermocyclers for real-time PCR.</u> <u>2. Important to use PCR in conjunction with diagnosis of anaemia and blood smear because PCR can detect low level rickettsemia leading to misdiagnosis.</u>	<u>Carelli <i>et al.</i>, 2007</u>

10 N/A: not available.
11 ¹RLB is the reverse line blot test.

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Appendix 5: Bovine anaplasmosis
Intended purpose of test: prevalence of infection – surveillance

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>CAT</u> <u>±</u>	<u>Serum</u> <u>Lysates of</u> <u><i>A. marginale</i></u> <u>isolated from red</u> <u>blood cells.</u>	<u>Reference test</u> <u>was blood smear.</u> <u>DSe 84.1¹-100²%</u> <u>Dsp 97.9¹-98.6²%</u>	<u>48 cattle raised in</u> <u>anaplasmosis free region.</u> <u>82 animals from endemic</u> <u>region.¹</u> <u>86 sera from experimentally</u> <u>infected cattle and 183 sera</u> <u>from <i>A. marginale</i> free area²</u>	<u>See references</u>	<u>1. Can be done in field or in</u> <u>the laboratory</u>	<u>1. Antigen derived from</u> <u>infected cattle are difficult to</u> <u>produce and standardise.</u> <u>2. May have false negative</u> <u>and false positive results.</u> <u>3. Variation between tests</u> <u>depending on environmental</u> <u>conditions and the</u> <u>laboratory.</u>	<u>¹Gonzalez <i>et al.</i>, 1978.</u> <u>²Molloy <i>et al.</i>, 1999.</u>
<u>C-ELISA</u> <u>+++</u> <u>Bovine</u>	<u>Serum</u> <u>rMSP5-GST</u>	<u>Reference tests</u> <u>were nested PCR</u> <u>and IFAT.</u> <u>Dsp = 99.7%</u> <u>Dse = 100%</u> <u>30% inhibition as</u> <u>determined by</u> <u>ROC analysis.</u>	<u>1. 358 known non-infected</u> <u>cattle from dairy herds</u> <u>maintained in tick free barns</u> <u>and no clinical history of</u> <u>clinical anaplasmosis.</u> <u>2. 135 known positive sera as</u> <u>defined by nested PCR.</u> <u>3. Intra-test comparison with</u> <u>163 diagnostic samples with</u> <u>possible false positives based</u> <u>on rMSP5-GST C-ELISA. Test</u> <u>positive confirmation done with</u> <u>IFAT.</u>	<u>See reference</u>	<u>1. Updated version with</u> <u>improved specificity.</u> <u>2. High sensitivity, detects</u> <u>persistently infected</u> <u>animals.</u> <u>3. Commercially available.</u> <u>4. Uses a standardised</u> <u>antigen.</u> <u>5. Target antigen is highly</u> <u>conserved among</u> <u><i>A. marginale</i> strains, thus</u> <u>detects infection with all</u> <u>strains of <i>A. marginale</i>.</u> <u>6. Rapid.</u>	<u>1. Does not differentiate</u> <u>between infection with</u> <u><i>A. marginale</i> and <i>A.</i></u> <u><i>centrale</i>.</u> <u>2. May cross react with anti-</u> <u><i>Ehrlichia</i> antibodies.</u> <u>3. May not be readily</u> <u>available in all countries.</u> <u>4. Requires a microplate</u> <u>absorbance reader.</u> <u>5. Low percent of false</u> <u>positive results</u>	<u>Chung <i>et al.</i>, 2014.</u>
<u>IFAT++</u> <u>Bovine</u>	<u>Serum</u> <u>Glass slides with</u> <u>RBCs infected</u> <u>with <i>A. marginale</i></u>	<u>Reference test</u> <u>was blood.</u> <u>DSe 97.6%</u> <u>Dsp 89.6%</u>	<u>1. 48 cattle raised in</u> <u>anaplasmosis free region.</u> <u>2. 82 animals from endemic</u> <u>region.</u>	<u>See references</u>	<u>1. Antigen is relatively easy</u> <u>to produce and store.</u> <u>2. Does not require many</u> <u>reagents.</u>	<u>1. Relatively high false</u> <u>positive rate.</u> <u>2. Time consuming and</u> <u>labour intensive</u> <u>so not suitable for high</u> <u>throughput.</u> <u>3. Requires fluorescent</u> <u>microscope and blood</u> <u>smears with high</u> <u>rickettsemia.</u>	<u>Gonzalez <i>et al.</i>, 1978</u>

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Appendix 6: Bovine anaplasmosis
Intended purpose of test: Immune status of individual animals

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population used to measure accuracy</u>	<u>Validation report</u>	<u>Advantages: expert opinion</u>	<u>Disadvantages: expert opinion</u>	<u>References</u>
<u>NA detection by (real-time) RT-PCR +++</u>	<u>Ear notch (skin), blood, milk</u>	<u>Performance has been demonstrated under field conditions in large control programs</u>	<u>Whole Swiss, German and Irish cattle populations</u>	<u>See references</u>	<ul style="list-style-type: none"> - <u>Very sensitive</u> - <u>Rapid</u> - <u>High-throughput</u> - <u>Well established internationally</u> - <u>Detects assay-dependent all BVDV species</u> - <u>Allows assay-dependent for differentiation of BVDV types 1 and 2</u> - <u>Detects persistent and transient infection</u> - <u>Proficiency panel of different Pestivirus strains available</u> - <u>Detection of viral RNA in skin biopsy samples unaffected by maternally-derived antibodies, therefore allows for identification of persistently infected animals early in life</u> - <u>Successfully applied in ongoing or completed control programmes</u> 	<ul style="list-style-type: none"> - <u>Possibility for contamination at sample collection or in laboratory, leading to false positive results</u> - <u>Needs specialised equipment</u> - <u>Detection of viral RNA does not imply per se that infectious virus is present</u> 	<ul style="list-style-type: none"> - <u>Presi & Heim (2010). <i>Vet. Microbiol.</i>, 142, 137–142</u> - <u>Schweizer et al. (2021) <i>Front. Vet. Sci.</i>, 8, 702730</u> - <u>Wernike et al. (2017). <i>Pathogens</i>, 6 (4)</u> - <u>Graham et al. (2021) <i>Front. Vet. Sci.</i>, 8, 674557</u>
<u>Antibody detection by ELISA +++</u>	<u>Bulk milk, blood</u>	<u>DSe and DSp differs based on the ELISA used (commercial/in-house) and the antibodies being tested (e.g. antibodies against structural (E2) and</u>			<ul style="list-style-type: none"> - <u>Simple to perform and cost-effective</u> - <u>Milk collection is non-invasive method with potential for herd screening with tank/bulk milk samples</u> 	<ul style="list-style-type: none"> - <u>Some cross-reactivity with vaccines and other pestiviruses</u> - <u>PI animal will usually be seronegative</u> - <u>Bulk milk from herd excludes males, non-lactating or young stock</u> 	<ul style="list-style-type: none"> - <u>Beauudeau et al. (2001). <i>Vet. Microbiol.</i>, 80, 329–337</u> - <u>Lanyon et al. (2013). <i>Aust. Vet. J.</i>, 91, 52–56.</u>

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population used to measure accuracy</u>	<u>Validation report</u>	<u>Advantages: expert opinion</u>	<u>Disadvantages: expert opinion</u>	<u>References</u>
		non-structural (NS2-3) proteins.			- Bulk milk sensitive indicator for PI in herd		
<u>Antigen detection by ELISA +++</u>	<u>Serum, whole blood, skin biopsy</u>	<u>DSe 67–100% and DSp 98.8–100% relative to virus isolation reported</u>			<u>Relatively simple to perform, rapid, can be cost-effective (when compared to virus isolation and PCR) and suitable for high-throughput applications. There is no need for cell culture facility.</u>	<u>Maternal antibodies in colostrum may interfere with testing for antigen in serum using ELISA in calves. Ear notch samples are less affected. PI calves <i>in utero</i> defies detection.</u>	<u>Lanyon <i>et al.</i> (2013). <i>Vet. J.</i> 199, 201–209;</u>
<u>Virus isolation +</u>	<u>Serum, whole blood</u>	<u>Considered (historically) reference test; DSe <90% compared with real-time RT-PCR ; DSp ~100%</u>	<u>N/A</u>	<u>Historical information with no formal validation</u>	<u>- High degree of specificity - Identifies presence of infectious virus</u>	<u>- Requires specialised cell culture capabilities and access to BVDV free materials - Reduced sensitivity in presence of maternally-derived antibodies</u>	<u>N/A</u>
<u>Virus neutralisation test +</u>	<u>Serum</u>	<u>DSe & DSp both extremely high, both >99%. Historical reference serological test.</u>	<u>N/A</u>	<u>Historical information with no formal validation</u>	<u>Very high specificity</u>	<u>- ASe can vary depending on virus strain used - Requires cell culture, good quality samples - Labour intensive, takes 5 days to obtain results - Expensive</u>	<u>N/A</u>

18 N/A: not available

Annexe 11. Chapter 3.4.7. 'Bovine viral diarrhoea'

CHAPTER 3.4.7.

BOVINE VIRAL DIARRHOEA

SUMMARY

Cattle of all ages are susceptible to infection with bovine viral diarrhoea (BVD) viruses, including Pestivirus bovis (commonly known as BVDV type 1 (Pestivirus bovis), Pestivirus tauri (BVDV type 2 (Pestivirus tauri), and Pestivirus brazilense (BVDV type 3 (Pestivirus brazilense) (or Hobi-like pestiviruses (type 3 [Pestivirus brazilense]). 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 prolonged and persistent testicular infection and excrete virus in semen for prolonged periods, perhaps indefinitely.

Detection of the agent: BVDV is a pestivirus in the family Flaviviridae and is closely related to classical swine fever virus (Pestivirus suis) and ovine border disease viruses (Pestivirus ovis). BVD viruses are classified into the distinct species: Pestivirus bovis (commonly known as BVDV type 1), Pestivirus tauri (BVDV type 2) and Pestivirus brazilense (BVDV type 3 or Hobi-like pestivirus). ~~The two genotypes (types 1 and 2) are classified as separate species in the genus Pestivirus. A third putative genotype, BVDV type 3, has also recently been proposed.~~ Although both cytopathic and non-cytopathic biotypes of Pestivirus bovis and P. tauri BVDV type 1 and type 2 exist, 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 usually 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.

44 *The testing of paired (acute and convalescent samples) should be done a minimum of 21*
45 *days apart and samples should be tested concurrently in the same assay. Enzyme-linked*
46 *immunosorbent assays and the virus neutralisation test are the most widely used.*

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

57 A. INTRODUCTION

58 1. Impact of the disease

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

73 Infections of the breeding female may result in conception failure or embryonic and fetal infection which
74 results in abortions, stillbirths, teratogenic abnormalities or the birth of PI calves. Persistently viraemic
75 animals may be born as weak, unthrifty calves or may appear as normal healthy calves and be
76 unrecognised clinically for a long time. However, PI animals have a markedly reduced life expectancy,
77 with a high proportion dying before reaching maturity. Infrequently, some of these animals may later
78 develop mucosal disease with anorexia, gastrointestinal erosions, and profuse diarrhoea, invariably
79 leading to death. Mucosal disease can arise only in PI animals. It is important to avoid the trade of
80 viraemic animals. It is generally considered that serologically positive, non-viraemic cattle are 'safe',
81 providing that they are not pregnant. However, a small proportion of persistently viraemic animals may
82 produce antibodies to some of the viral proteins if they are exposed to another strain of BVDV that is
83 antigenically different to the persisting virus. Consequently, seropositivity cannot be completely equated
84 with 'safety'. Detection of PI animals must be specifically directed at detection of the virus or its
85 components (RNA or antigens). Latent infections generally do not occur following recovery from acute
86 infection. However, semen collected from bulls during an acute infection is likely to contain virus during
87 the viraemic period and often for a short time afterwards. Although extremely rare, some recovered bulls
88 may have a prolonged and persistent testicular infection and excrete virus in semen, perhaps indefinitely
89 (Read et al., 2020).

90 While BVDV strains are predominantly pathogens of cattle, interspecies transmission can occur
91 following close contact with sheep, goats or pigs. Infection of pregnant small ruminants or pigs with
92 BVDV can result in reproductive loss and the birth of PI animals. BVDV infections have been reported
93 in both New World and Old World camelids. Additionally, strains of border disease virus (BDV) have
94 infected cattle, resulting in clinical presentations indistinguishable from BVDV infection. The birth of
95 cattle PI with BDV and the subsequent development of mucosal disease have also been described.

96 Whilst BVDV and BDV have been reported as natural infections in pigs, the related virus of classical
97 swine fever does not naturally infect ruminants.

98 Although ubiquitous, control of BVDV can be achieved at the herd level, and even at the national level,
99 as evidenced by the progress towards eradication made in many European countries (Moennig *et al.*,
100 2005; Schweizer *et al.*, 2021).

101 2. The causal agent

102 Bovine viral diarrhoea virus (BVDV) is a single linear positive-stranded RNA virus in the genus *Pestivirus*
103 of the family *Flaviviridae*. The genus contains a number of species including *Pestivirus bovis* the two
104 genotypes of bovine viral diarrhoea virus (BVDV) (types 1 [*Pestivirus bovis*], and 2 [*Pestivirus tauri*]
105 (BVDV type 2) and 3 [*Pestivirus brasilense*] (BVDV type 3) and the closely related classical swine fever
106 (*Pestivirus suis*) and ovine border disease viruses (*Pestivirus ovis*) (Postler *et al.*, 2023). Viruses in these
107 genotypes-pestivirus species show considerable antigenic difference from each other and, within the
108 type 1 and type 2 species *Pestivirus bovis* and *P. tauri*, BVDV isolates exhibit considerable biological
109 and antigenic diversity. Within the two BVDV genotypes-species *Pestivirus bovis* and *P. tauri*, further
110 subdivisions are discernible by genetic analysis (Vilcek *et al.*, 2001). The two genotypes-species may
111 be differentiated from each other, and from other pestiviruses, by monoclonal antibodies (MAbs) directed
112 against the major glycoproteins E2 and ERNS or by genetic analysis. Reverse-transcription polymerase
113 chain reaction (RT-PCR) assays enable virus typing direct from blood samples (Letellier & Kerkhofs,
114 2003; McGoldrick *et al.*, 1999). Type 1 viruses are generally more common although the prevalence of
115 type 2 strains can be high in North America. BVDV of both genotypes-species (*Pestivirus bovis* and *P.*
116 *tauri*) may occur in non-cytopathic and cytopathic forms (biotypes), classified according to whether or
117 not microscopically apparent cytopathology is induced during infection of cell cultures. Usually, it is the
118 non-cytopathic biotype that circulates freely in cattle populations. Non-cytopathic strains are most
119 frequently responsible for disease in cattle and are associated with enteric and respiratory disease in
120 any class of cattle or reproductive and fetal disease following infection of a susceptible breeding female.
121 Infection may be subclinical or extend to severe fatal disease (Brownlie, 1985). Cytopathic viruses are
122 encountered in cases of mucosal disease, a clinical syndrome that is relatively uncommon and involves
123 the 'super-infection' of an animal that is PI with a non-cytopathic virus by a closely related cytopathic
124 strain. The two virus biotypes found in a mucosal disease case are usually antigenically closely related
125 if not identical. Type 2 viruses are usually non-cytopathic and have been associated with outbreaks of
126 severe acute infection and a haemorrhagic syndrome. However some type 2 viruses have also been
127 associated with a disease indistinguishable from that seen with the more frequently isolated type 1
128 viruses. Further, some type 1 isolates have been associated with particularly severe and fatal disease
129 outbreaks in adult cattle. Clinically mild and inapparent infections are common following infection of non-
130 pregnant animals with either genotype-virus species.

131 There is an increasing awareness of an "atypical" or "HoBi-like" pestivirus – a putative BVDV type 3
132 *Pestivirus brasilense* H-strains are also associated with clinical disease in cattle, but they appear mainly
133 restricted to South American and Asian cattle populations, in cattle, also associated with clinical disease
134 (Bauermann *et al.*, 2013; Chen *et al.*, 2021), but its distribution is presently unclear. These viruses are
135 readily detected by proven pan-reactive assays such as real-time RT-PCR. Some commercial antigen
136 ELISAs (enzyme-linked immunosorbent assays) have been shown to detect these strains (Bauermann
137 *et al.*, 2012); generally virus isolation, etc., follows the same principles as for *Pestivirus bovis* (BVDV
138 type 1 (*Pestivirus bovis*) and *Pestivirus tauri* (BVDV type 2 (*Pestivirus tauri*)). It should be noted however,
139 that antibody ELISAs vary in their ability to detect antibody to *Pestivirus brasilense* (BVDV type 3
140 (*Pestivirus brasilense*)) and vaccines designed to protect against BVDV type 1 and BVDV type 2 may
141 not confer full protection against infection with these novel-pestiviruses (Bauermann *et al.*, 2012; 2013).

142 3. Pathogenesis

143 3.1. Acute infections

144 Acute infections with BVDV are encountered more frequently in young animals, and may be
145 clinically inapparent or associated with fever, diarrhoea (Baker 1995), respiratory disease and
146 sometimes sudden death. The severity of disease may vary with virus strain and the involvement
147 of other pathogens (Brownlie, 1990). In particular, outbreaks of a severe form of acute disease
148 with haemorrhagic lesions, thrombocytopenia and high mortality have been reported
149 sporadically from some countries (Baker, 1995; Bolin & Ridpath, 1992). Infection with type 2

150 viruses (*Pestivirus tauri*) in particular has been demonstrated to cause altered platelet function.
151 During acute infections there is a brief viraemia for 7–10 days and shedding of virus can be
152 detected in nasal and ocular discharges. There may also be a transient leukopenia,
153 thrombocytopenia or temperature response, but these can vary greatly among animals. Affected
154 animals may be predisposed to secondary infections with other viruses and bacteria. Although
155 BVDV may cause a primary respiratory disease on its own, the immunosuppressive effects of
156 the virus exacerbate the impact of this virus. BVDV is one of the major pathogens of the bovine
157 respiratory disease complex in feedlot cattle and in other intensive management systems such
158 as calf raising units.

159 Infection of breeding females immediately prior to ovulation and in the first few days after
160 insemination can result in conception failure and early embryonic loss (McGowan & Kirkland,
161 1995). Cows may also suffer from infertility, associated with changes in ovarian function and
162 secretions of gonadotropin and progesterone (Fray *et al.*, 2002). Bulls may excrete virus in
163 semen for a short period during and immediately after infection and may suffer a temporary
164 reduction of fertility. Although the virus level in this semen is generally low it can result in
165 reduced conception rates and be a potential source of introduction of virus into a naive herd
166 (McGowan & Kirkland, 1995).

167 **3.2. In-utero infections**

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

186 **3.3. Persistent infections**

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

196 **3.4. Mucosal disease**

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

204 may be the first indication in a herd that BVDV infection is present and should lead to more in
205 depth investigation and intervention.

206 **3.5. Semen and embryos**

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

221 Biological materials used for *in-vitro* fertilisation techniques (bovine serum, bovine cell
222 cultures) have a high risk of contamination and should be screened for BVDV. Incidents of
223 apparent introduction of virus via such techniques have highlighted this risk. It is considered
224 essential that serum supplements used in media should be free of contaminants as detailed
225 in Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials*
226 *intended for veterinary use*, using techniques described in Section B.3.1.1 of this chapter.

227 **4. Approaches to diagnosis and sample collection**

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

232 **4.1. Acute infections**

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

246 Confirmation that an abortion, stillbirth or perinatal death is caused by BVDV is often difficult
247 to establish because there can be a long delay between initial infection and death or expulsion
248 of the fetus. Sampling should take into consideration the need to detect either viral
249 components or antibodies. Spleen and lung are preferred samples for virus detection while
250 pericardial or pleural fluids are ideal samples for serology. The stomach of newborn calves
251 should be checked to confirm that sucking has not occurred. While virus may be isolated from
252 fetal tissue in some cases, emphasis should be placed on the detection of viral antigen by
253 ELISA or RNA by real-time RT-PCR. For serology, both ELISAs and virus neutralisation test
254 (VNT) are suitable though sample quality and bacterial contamination may compromise the
255 ability to detect antibodies by VNT. Maternal serology, especially on a group of animals, can
256 be of value, with the aim of determining whether there has been recent infection in the group.

257 A high antibody titre (>1/1000) to BVDV in maternal serum is suggestive of fetal infection and
258 is probably due to the fetus providing the dam with an extended exposure to virus.

259 **4.2. Persistent infections**

260 In the past, identification of PI animals relied heavily on the use of virus isolation in cell cultures.
261 However, antigen detection ELISAs and real-time RT-PCR assays, each with relatively high
262 sensitivity, are widely used for the detection of viral antigens or RNA in both live and dead
263 animals. Virus isolation aimed at the detection of non-cytopathic BVDV in blood is also used,
264 while in some countries, the virus has been identified by immunohistochemistry (IHC). Skin
265 samples have been collected from live animals while a wide range of tissues from dead
266 animals are suitable. Both virus isolation and IHC are labour intensive and costly and can be
267 technically demanding. Virus isolation from blood can be confounded by the presence of
268 maternal antibodies to BVDV in calves less than 4–5 months of age (diagnostic gap). Also for
269 antigen detection ELISAs and flow cytometry from blood or blood leukocytes, there are
270 restrictions that limit when animals that ingested colostrum that contains antibodies to against
271 BVDV can be reliably tested. In older animals with persistent viraemia infection, low levels of
272 antibody may be present due to their ability to seroconvert to strains of BVDV (including
273 vaccines) antigenically different to the persisting virus (Brownlie, 1990). Bulk (tank) or
274 individual milk samples have been used to monitor dairy herds for the presence of a PI animal.
275 Antigen ELISA, real-time PCR and virus isolation have all been used. To confirm a diagnosis
276 of persistent infection, animals should be retested after an interval of at least 3 weeks by
277 testing of blood samples for the presence of the virus and for ~~evidence~~ absence of
278 seroconversion. Care should be taken with retesting of skin samples as it has been shown
279 that, in some acute cases, viral antigen may persist for many weeks in skin (Cornish *et al.*,
280 2005).

281 **4.3. Mucosal disease**

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

287 **4.4. Reproductive materials**

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

300

B. DIAGNOSTIC TECHNIQUES

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

Method	Purpose					
	Population freedom from infection ^(a)	Individual animal freedom from infection prior to movement ^(b)	Contribute to eradication policies ^(c)	Confirmation of clinical cases ^(d)	Prevalence of infection – surveillance ^(e)	Immune status in individual animals or populations (post-vaccination) ^(f)
Detection of the agent^(g)						
Virus isolation	+	++ +	++	++ +	–	–
Antigen detection by ELISA	+++ _±	+++	+++	+++	+++	–
Antigen detection by IHC	–	–	–	++	–	–
NA detection by real-time RT-PCR	+++	+++	+++	+++	+++	–
Detection of immune response						
Antibody detection by ELISA	+++	++	+++	– <u>+(g)</u>	+++	+++
VN	+	++-+	++	–	+	+++

302

Key: +++ = recommended for this purpose; ++ recommended but has limitations;

303

+ = suitable in very limited circumstances; – = not appropriate for this purpose.

304

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.

305

^(a)See Appendix 1 of this chapter for justification table for the scores giving to the tests for this purpose.

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^(b)See Appendix 2 of this chapter for justification table for the scores giving to the tests for this purpose.

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^(c)See Appendix 3 of this chapter for justification table for the scores giving to the tests for this purpose.

308

^(d)See Appendix 4 of this chapter for justification table for the scores giving to the tests for this purpose.

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^(e)See Appendix 5 of this chapter for justification table for the scores giving to the tests for this purpose.

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^(f)See Appendix 6 of this chapter for justification table for the scores giving to the tests for this purpose.

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^(g)A combination of agent detection methods applied on the same clinical sample is recommended.

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1. Detection of the agent

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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 seronegative animals are not undergoing an acute infection or, to establish the serological status of donor bulls. Due to their variable sensitivity without prior virus amplification, procedures such as IHC or *in-situ* hybridisation (ISH) directly on tissues are not considered to be suitable for certification for freedom from BVDV for international trade purposes. In contrast, immune-staining is an essential component of virus isolation in cell culture to detect the presence of non-cytopathic strains of BVDV which predominate in field infections.

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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 MA-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 WOA Reference Laboratories for BVDV that can assist with relevant information; the reference laboratories for classical swine fever could also be approached to offer some advice.

331

1.1. Virus isolation

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

341 The virus may be isolated in a number of bovine monolayer cell cultures (e.g. kidney, lung,
342 testis or turbinate). In some instances, ovine cells are also suitable. Primary or secondary
343 cultures can be frozen as cell suspensions in liquid nitrogen. These can then be tested over a
344 series of passages, or seeded to other susceptible cells and checked for freedom from
345 contaminants and to evaluate their sensitivity compared to an approved batch of cells before
346 routine use. Such problems may be reduced by the use of continuous cell lines, which can be
347 obtained BVD-free, however, their BVDV-free status and susceptibility must be monitored
348 regularly. Continuous cells should be used under a 'seed lot' system where they are only used
349 over a limited passage range, within which they have been shown to have acceptable
350 sensitivity to BVDV infection. Although particular continuous cell lines are considered to be
351 appropriate for use for BVDV isolation, there can be significant variation in batches of cells
352 from different sources due to differing passage histories so their suitability must still be
353 confirmed before routine use.

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

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

387 including higher sensitivity and the potential to be completed within a few hours rather than
388 weeks for virus isolation.

389 There are many variations of procedure in use for virus isolation. All should be optimised to
390 give maximum sensitivity of detection of a standard virus preparation. All biological
391 components used for cell culture should be screened and shown to be free of both BVDV and
392 antibodies to BVDV. Cell cultures (whether primary or continuous lines) should be regularly
393 checked to confirm that they maintain maximum susceptibility to virus infection. Depending on
394 the specimen type and purpose for testing, virus isolation is likely to require one or more
395 passages in cell cultures. While PI animals can be readily identified by screening blood or
396 serum with one passage, semen should be routinely cultured for three passages and biological
397 products such as fetal bovine serum up to five times (original inoculation plus four passages).
398 Conventional methods for virus isolation are used, with the addition of a final immune-staining
399 step (immunofluorescence or, more frequently, peroxidase staining) to detect growth of non-
400 cytopathic virus. Thus, tube cultures should include flying cover-slips, while microplate cultures
401 can be fixed and labelled directly in the plate. Examples are given below. Alternatively, culture
402 supernatant from the final passage can be screened by real-time RT-PCR (see below).

403 **1.1.1. Microplate immunoperoxidase method for mass screening for virus detection in** 404 **serum samples (Meyling, 1984)**

- 405 i) 10–25 µl of the serum sample is placed into each of four wells of a 96-well tissue-
406 culture grade microplate. This is repeated for each sample. Known positive and
407 negative controls are included.
- 408 ii) 100 µl of a cell suspension at the appropriate concentration (usually about
409 150,000 cells/ml) in medium without fetal calf serum (FCS) is added to all wells.
410 *Note:* the sample itself acts as the cell-growth supplement. If testing samples other
411 than serum, use medium with 10% FCS that is free of antibodies to ruminant
412 pestiviruses.
- 413 iii) The plate is incubated at 37°C for 4 days, either in a 5% CO₂ atmosphere or with
414 the plate sealed.
- 415 iv) Each well is examined microscopically for evidence of cytopathology (cytopathic
416 effect or CPE), or signs of cytotoxicity.
- 417 v) The cultures are frozen briefly at approximately –80°C and 50 µl of the culture
418 supernatant is passaged to new cell cultures, repeating steps 31.1.1.i to iv above.
- 419 vi) The cells are then fixed and stained by one of two methods:
- 420 ● **Paraformaldehyde**
- 421 a) Add 200 µl of a 1/10 dilution of formaldehyde solution (approximately 3%
422 concentration) to the plate and leave at room temperature for 10 minutes.
- 423 b) The contents of the plate are then discarded and the plate is washed.
- 424 c) Wash plates 5 times with 0.05% Tween 20 in water (an automatic microplate
425 washer can be used with a low pressure and speed setting).
- 426 d) To each well add 50 µl of an antiviral antibody at the appropriate dilution (prepared
427 in phosphate buffered saline/ PBS containing 1% gelatin) and incubate for 60–
428 90 minutes at 37°C in a humidified chamber.
- 429 e) Wash plates five times as in step c).
- 430 f) Dilute the appropriate peroxidase conjugated antiserum to the optimum dilution in 1%
431 gelatin/PBS (e.g. peroxidase conjugated rabbit anti-mouse immunoglobulin when the
432 antiviral antibody is a mouse monoclonal). The optimum concentration should be
433 determined for each batch of conjugate by “checkerboard” titration against reference
434 positive and negative controls.
- 435 g) To each well of the microplate add 50 µl of the diluted peroxidase conjugate and
436 incubate for 90 minutes at 37°C in a humidified chamber.

- 437 h) Wash plates five times as in step c).
- 438 i) “Develop” the plate by adding 3-amino-9-ethyl carbazole (AEC) substrate (100
439 µl/well) and allowing to react for 30 minutes at room temperature.
- 440 j) Add 100 µl of PBS to each well and add a lid to each plate.
- 441 k) Examine the wells by light microscopy, starting with the negative and positive control
442 wells. There should be no or minimal staining apparent in the cells that were
443 uninfected (negative control). The infected (positive control) cells should show a
444 reddish- brown colour in the cytoplasm.
- 445 ● **Acetone**
- 446 a) The plate is emptied by gentle inversion and rinsed in PBS.
- 447 b) The cells are fixed as follows: the plate is dipped into a bath of 20% acetone in
448 PBS, emptied immediately and then transferred to a fresh bath of 20% acetone in
449 PBS for 10 minutes. The plate is drained thoroughly and as much fluid as possible
450 is removed by tapping and blotting. The plate is dried thoroughly for at least 3 hours
451 at a temperature of 25–30°C (e.g. using radiant heat from a bench lamp). *Note:* the
452 drying is part of the fixation process.
- 453 c) The fixed cells are rinsed by adding PBS to all wells.
- 454 d) The wells are drained and the **antiviral** BVD antibody (50 µl) is added to all wells at
455 a predetermined dilution in PBS containing 1% Tween 80 (PBST) and 5% horse
456 serum or 1% gelatin. (Horse serum or gelatin may be added to reduce nonspecific
457 staining.)
- 458 e) Incubate at 37°C for 15 minutes.
- 459 f) Empty the plate and wash three times in PBST.
- 460 g) Drain and add the appropriate anti-species serum conjugated to peroxidase at a
461 predetermined dilution in PBST (50 µl per well) for 15 minutes at 37°C.
- 462 h) Empty the plate and wash three times in PBST.
- 463 i) Rinse the plate in distilled water. Ensure all fluid is tapped out from the plate.
- 464 j) Add freshly prepared hydrogen peroxide substrate with a suitable chromogen, e.g.
465 3-amino-9-ethyl carbazole (AEC).
- 466 An alternative substrate can be made, consisting of 9 mg diaminobenzidine
467 tetrahydrochloride and 6 mg sodium perborate tetrahydrate dissolved in 15 ml of
468 PBS. Though the staining is not quite so intense, these chemicals have the
469 advantage that they can be shipped by air.
- 470 k) The plate is examined microscopically. Virus-positive cells show red-brown
471 cytoplasmic staining.
- 472 Alternative methods for fixation of the cells may be used and include the use of heat
473 (see Chapter 3.8.3 *Classical swine fever*, Section B.2.2.1.viii). These should be first
474 evaluated to ensure that the capacity to detect viral antigen is not compromised.

475 1.1.2. Tube method for tissue or buffy coat suspensions

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

- 478 i) Tissue samples are ground up and a 10% suspension in culture medium is made.
479 This is then centrifuged to remove the debris.
- 480 ii) Test tube cultures with newly confluent or subconfluent monolayers of susceptible
481 bovine cells are inoculated with 0.1 ml of the sample. The culture is left to adsorb
482 for 1 hour at 37°C.

- 483 iii) The culture is washed with 1 ml of medium; this is then discarded and 1 ml of
484 culture maintenance medium is added.
- 485 iv) The culture is incubated for 4–5 days at 37°C and examined microscopically for
486 evidence of CPE or signs of cytotoxicity.
- 487 v) The culture should then be frozen and thawed for passage to fresh cultures for one
488 or preferably two more passages (including the culture inoculated for the final
489 immunostaining). At the final passage, after freeze–thaw the tissue culture fluid is
490 harvested and passaged on to microtitre plates for culture and staining by the
491 immunoperoxidase method (see section B.31.1.1 above) or by the
492 immunofluorescent method. For immunofluorescence, cover-slips are included in
493 the tubes and used to support cultured cells. At the end of the culture period, the
494 cover slips are removed, fixed in 100% acetone and stained with an
495 immunofluorescent conjugate to BVDV. Examine the cover slips under a
496 fluorescent microscope for diffuse, cytoplasmic fluorescence characteristic of
497 pestiviruses. Alternatively, culture supernatant from the final passage can be
498 screened by real-time RT-PCR (see below).

499 1.1.3. Virus isolation from semen

500 The samples used for the test are, typically, extended bovine semen or occasionally
501 raw semen. Semen samples should be transported to the laboratory in liquid nitrogen,
502 or on dry ice. The samples should be stored in liquid nitrogen or at lower than –70°C
503 (for long-term storage) or 4°C (for short-term storage of not more than 1–2 days). The
504 receiving laboratory should document the condition under which samples are received.
505 Raw semen is generally cytotoxic and should be prediluted (e.g. 1/10 in BVDV free
506 bovine serum) before being added to cell cultures. At least 0.1 ml of raw semen should
507 be tested with three passages in cell culture. Toxicity may also be encountered with
508 extended semen. For extended semen, an approximation should be made to ensure
509 that the equivalent of a minimum of 0.1 ml raw semen is examined (e.g. a minimum of
510 1.0 ml extended semen). If toxicity is encountered, multiple diluted samples may need
511 to be tested to reach a volume equivalent to 0.1 ml raw semen (e.g. 5 × 1 ml of a sample
512 of extended semen that has been diluted 1/5 to reduce toxicity). A suggested method is
513 as follows:

- 514 i) Dilute 200 µl fresh semen in 1.8 ml bovine serum containing antibiotics. This can
515 be the same serum as is being used for supplementing the cell cultures, and must
516 be shown to be free from antibodies ~~to~~ against BVDV.
- 517 ii) Mix vigorously and leave for 30 minutes at room temperature.
- 518 iii) Inoculate 1 ml of the semen/serum mixture into a monolayer of susceptible cells
519 (see virus isolation from tissue above) in cell culture tubes or a six-well tissue
520 culture plate.
- 521 iv) Incubate the cultures for 1 hour at 37°C.
- 522 v) Remove the mixture, wash the monolayer several times with maintenance medium
523 and then add new maintenance medium to the cultures.
- 524 vi) Include BVDV negative and positive controls in the test. Special caution must be
525 taken to avoid accidental contamination of test wells by the positive control, for
526 example always handling the positive control last.
- 527 vii) Observe plates microscopically to ensure freedom from contamination and
528 cytotoxicity. No cytopathology is expected as a result of BVDV infection but other
529 viruses such as BHV-1 could be inadvertently isolated.
- 530 viii) After 5–7 days, the cultures are frozen at or below approximately –70°C and
531 thawed, clarified by centrifugation, and the supernatant used to inoculate fresh
532 monolayers.
- 533 ix) At the end of the second passage, the supernatant from the freeze-thaw
534 preparation should be passaged onto cultures in a suitable system for
535 immunoperoxidase staining or other antigen detection or by real-time RT-PCR after

536 5 days of culture. This is most readily achieved in 96 well microplates. The sample
537 is considered to be negative, if there is no evidence of viral antigen or BVDV RNA
538 detected.

539 1.2. Nucleic acid detection

540 Conventional gel-based RT-PCR has in the past been used for the detection of BVD viral RNA
541 for diagnostic purposes. A multiplex RT-PCR has been used for the simultaneous amplification
542 and typing of virus from cell culture, or direct from blood samples. However, gel-based RT-
543 PCR has the disadvantage that it is relatively labour intensive, expensive and prone to cross
544 contamination. These problems had been markedly reduced following the introduction of
545 probe-based real-time or quantitative RT-PCR methods. Nevertheless, stringent precautions
546 should still be taken to avoid nucleic acid contamination in the test system and general
547 laboratory areas where samples are handled and prepared (see Chapter 1.1.6 *Principles and*
548 *methods of validation of diagnostic assays for infectious diseases* and Chapter 2.2.3
549 *Development and optimisation of nucleic acid assays*). These assays have even higher
550 sensitivity than gel-based RT-PCR and can be completed in a few hours. They are in
551 widespread use for the diagnosis of infectious diseases, allowing the direct detection of viral
552 RNA from a wide range of specimens including serum, whole blood, tissues, milk and semen.
553 The high analytical sensitivity allows the adoption of strategies to screen pools of individual
554 samples or testing of bulk tank milk. By using this approach, the presence of one or more PI
555 animals can be identified in herds containing several hundred cows. However, it is not
556 appropriate to pool blood samples taken from calves between day 7 and 40 of life, when
557 colostrum that contains antibodies to against BVDV was ingested. During this time the
558 sensitivity of PCR can be reduced and infected animals escape detection. In contrast, the
559 detection of viral RNA in skin biopsy samples remains unaffected (Fux & Wolf, 2012). Although
560 slightly more expensive than immunostaining methods, real-time RT-PCR is a quick and
561 reliable method that can also be used to screen culture supernatant from the final passage of
562 cell cultures. While real-time RT-PCR has very high sensitivity and can be applied to the
563 screening of biological materials used for vaccine manufacture, caution is needed in the
564 interpretation of results, as the detection of viral RNA does not imply *per se* that infective virus
565 is present. Real-time RT-PCR assays based on fluorescent-labelled DNA probes can also be
566 used to differentiate pestiviruses (e.g. McGoldrick *et al.*, 1999).

567 Primers for the assay should be selected in highly conserved regions of the genome, ideally
568 the 5'-noncoding region, or the NS3 (p80 gene). There are published assays that are broadly
569 reactive across the pestivirus genus, detecting all BVDV types (*Pestivirus bovis*, *tauri* and
570 *brazilense*), CSFV (*Pestivirus suis*), some strains of BDV (*Pestivirus ovis*) and most of the
571 several 'atypical' pestiviruses (e.g. Hoffman *et al.*, 2006). A sensitive broadly reactive assay is
572 recommended for diagnostic applications because interspecies transfer of different
573 pestiviruses is occasionally encountered. When further identification of the specific virus is
574 required, pestivirus species-specific assays can be applied to further type the virus. It is
575 important to thoroughly optimise all aspects of the real-time RT-PCR assay, including the
576 nucleic acid extraction and purification. Optimal concentrations of Mg²⁺, primers, probe and
577 polymerase, and the cycling parameters need to be determined. However, fully formulated
578 and optimised 'ready to use' 'mastermixes' are now available commercially and only require
579 addition of optimised concentrations of primers and probe. Optimised cycling conditions are
580 often recommended for a particular mastermix.

581 A variety of commercially available nucleic acid purification systems are available in kit form,
582 and several can be semi-automated. Systems based on the capture and purification of RNA
583 using magnetic beads are in widespread use and allow rapid processing of large numbers of
584 samples. Specific products should be evaluated to determine the optimal kit for a particular
585 sample type and whether any preliminary sample processing is required. For whole blood
586 samples, the type of anticoagulant and volume of blood in a specimen tube is important. More
587 problems with inhibitors of the PCR reaction are encountered with samples collected into
588 heparin treated blood than EDTA. These differences are also exacerbated if the tube does not
589 contain the recommended volume of blood, thereby increasing the concentration of
590 anticoagulant in the sample. To identify possible false-negative results, it is recommended to
591 spike an exogenous ('internal control') RNA template into the specimen prior to RNA extraction

592 (e.g. Hoffman *et al.*, 2006). By the inclusion of PCR primers and probe specific to the
593 exogenous sequence, the efficiency of both the RNA extraction and also the presence of any
594 PCR inhibitors can be monitored. While valuable for all sample types, the inclusion of an
595 internal control is particularly desirable when testing semen and whole blood. When using an
596 internal control, extensive testing is necessary to ensure that PCR amplification of the internal
597 control does not compete with the diagnostic PCR and thus lower the analytical sensitivity (see
598 also chapter 1.1.6).

599 When it is suspected that a sample may contain substances that are adversely affecting either
600 the efficiency of RNA extraction or the real-time RT-PCR assay, modest dilution of the sample
601 in saline, cell culture medium or a buffer solution (e.g. phosphate buffered gelatin saline
602 [PBGS]) will usually overcome the problem. Dilution of a semen sample by 1/4 and whole
603 unclotted blood at 1/10 is usually adequate. As the real-time RT-PCR has extremely high
604 analytical sensitivity, dilution of the sample rarely has a significant impact on the capacity of
605 the assay to detect viral RNA when present.

606 1.2.1. Real-time polymerase chain reaction for BVDV detection in semen

607 Real-time RT-PCR has been shown to be extremely useful to screen semen samples
608 to demonstrate freedom from BVDV and, apart from speed, often gives superior results
609 to virus isolation in cell culture, especially when low virus levels are present, such as
610 may be found in bulls with a PTI. The real-time RT-PCR described here uses a pair of
611 sequence-specific primers for amplification of target D-RNA and a 5'-nuclease
612 oligoprobe for the detection of amplified products. The oligoprobe is a single, sequence-
613 specific oligonucleotide, labelled with two different fluorophores. The primers and probe
614 are available commercially and several different fluorophores options are available. This
615 pan-pestivirus real-time RT-PCR assay is designed to detect viral D-RNA of all strains
616 of BVDV types 1 (*Pestivirus bovis*) and BVDV₂ (*Pestivirus tauri*) and 3 (*Pestivirus*
617 *brazilense*) as well as BDV, CSFV (*Pestivirus suis*), some strains of BDV (*Pestivirus*
618 *ovis*) and most atypical pestiviruses. The assay selectively amplifies a 208 base pair
619 sequence of the 5' non-translated region (5' NTR) of the pestivirus genome. Details of
620 the primers and probes are given in the protocol outlined below.

621 i) Sample preparation, equipment and reagents

622 a) The samples used for the test are, typically, extended bovine semen or
623 occasionally raw semen. If the samples are only being tested by real-time RT-PCR,
624 it is acceptable for them to be submitted chilled, but they must still be cold when
625 they reach the laboratory. Otherwise, if a cold chain cannot be assured or if virus
626 isolation is being undertaken, the semen samples should be transported to the
627 laboratory in liquid nitrogen or on dry ice. At the laboratory, the samples should be
628 stored in liquid nitrogen or at lower than -70°C (for long-term storage) or 4°C (for
629 short-term storage of up to 7 days). *Note*: samples for virus isolation should not be
630 stored at 4°C for more than 1–2 days.

631 b) Due to the very high analytical sensitivity of real-time RT-PCR, much smaller
632 volumes of semen may be used. However, at least three straws (minimum 250 μl
633 each) from each collection batch of semen should be processed. The semen in the
634 three straws should be pooled and mixed thoroughly before taking a sample for
635 nucleic acid extraction.

636 c) A real-time PCR detection system, and the associated data analysis software, is
637 required to perform the assay. A number of real-time PCR detection systems are
638 available from various manufacturers. ~~Other equipment required for the test~~
639 ~~includes a micro-centrifuge, a chilling block, a micro-vortex, and micropipettes.~~ As
640 real-time RT-PCR assays are able to detect very small amounts of target nucleic
641 acid molecules, appropriate measures are required to avoid contamination, ~~;~~
642 ~~including dedicated and physically separated 'clean' areas for reagent preparation~~
643 ~~(where no samples or materials used for PCR are handled), a dedicated sample~~
644 ~~processing area and an isolated area for the PCR thermocycler and associated~~
645 ~~equipment. Each area should have dedicated reagents and equipment.~~
646 Furthermore, a minimum of one negative sample should be processed in parallel

647 to monitor the possibility of low level contamination. Sources of contamination may
648 include product carry-over from positive samples or, more commonly, from cross
649 contamination by PCR products from earlier work.

650 d) The real-time RT-PCR assay involves two separate procedures.

651 1) Firstly, BVDV RNA is extracted from semen using an appropriate
652 validated nucleic acid extraction method. Systems using magnetic beads
653 for the capture and purification of the nucleic acid are recommended. It
654 is also preferable that the beads are handled by a semi-automated
655 magnetic particle handling system.

656 2) The second procedure is the RT-PCR analysis of the extracted RNA
657 template in a real-time RT-PCR system.

658 ii) Extraction of RNA

659 RNA or total nucleic acid is extracted from the pooled (three straws collected at the
660 same time from the same animal) semen sample. Use of a commercially available
661 magnetic bead based extraction kit is recommended. However, the preferred kit
662 should be one that has been evaluated to ensure optimal extraction of difficult
663 samples (semen and whole blood). Some systems and kit protocols are sufficiently
664 refined that it is not necessary to remove cells from the semen sample. Prior to
665 extraction dilute the pooled semen sample 1/4 in phosphate buffered gelatin saline
666 (PBGS) or a similar buffered solution. Complete the RNA extraction by taking 50
667 µl of the diluted, pooled sample and add it to the sample lysis buffer. Some
668 commercial extraction kits may require the use of a larger volume. It has also been
669 found that satisfactory results are obtained by adding 25 µl of undiluted pooled
670 sample to sample lysis buffer. Complete the extraction by following the kit
671 manufacturer's instructions.

672 iii) Real-time RT-PCR assay procedure

673 a) Reaction mixture: There are a number of commercial real-time PCR amplification
674 kits available from various sources and the particular kits selected need to be
675 compatible with the real-time PCR platform selected. The required primers and
676 probes can be synthesised by various commercial companies. The WOH
677 Reference Laboratories for BVDV can provide information on suitable suppliers.

678 b) Supply and storage of reagents: The real-time PCR reaction mixture is normally
679 provided as a 2 × concentration ready for use. The manufacturer's instructions
680 should be followed for application and storage. Working stock solutions for primers
681 and probe are made with nuclease-free water at the concentration of 20 µM and
682 3 µM, respectively. The stock solutions are stored at -20°C and the probe solution
683 should be kept in the dark. Single-use or limited use aliquots can be prepared to
684 limit freeze-thawing of primers and probes and extend their shelf life.

685 c) Primers and probe sequences

686 Selection of the primers and probe are outlined in Hoffmann *et al.* (2006) and
687 summarised below.

688 *Forward:* BVD 190-F 5'-GRA-GTC-GTC-ART-GGT-TCG-AC
689 *Reverse:* V326 5'-TCA-ACT-CCA-TGT-GCC-ATG-TAC
690 *Probe:* TQ-pesti 5'-FAM-TGC-YAY-GTG-GAC-GAG-GGC-ATG-C-
691 TAMRA-3'

692 d) Preparation of reaction mixtures

693 The PCR reaction mixtures are prepared in a separate room that is isolated from
694 other PCR activities and sample handling. For each PCR test, appropriate controls
695 should be included. As a minimum, a no template control (NTC), appropriate
696 negative control (NC) and two positive controls (PC1, PC2) should be included.
697 The positive and negative controls are included in all steps of the assay from
698 extraction onwards while the NTC is added after completion of the extraction. The

699 PCR amplifications are carried out in a volume of 25 µl. The protocol described is
700 based on use of a 96 well microplate based system but other options using
701 microtubes are also suitable. Each well of the PCR plate should contain 20 µl of
702 reaction mix and 5 µl of sample as follows:

703	12.5 µl	2× RT buffer – from a commercial kit.
704	1 µl	BVD 190-F Forward primer (20 µM)
705	1 µl	V326 Reverse primer (20 µM)
706	1 µl	TQ-pesti Probe (3 µM)
707	2 µl	tRNA (40 ng/µl)
708	1.5 µl	<u>nuclease free</u> water
709	1 µl	25× enzyme mix
710	5 µl	sample (or controls – NTC, NC, PC1, PC2)

711 e) Selection of controls

712 NTC: usually consists of nuclease free water or tRNA in nuclease free water that
713 is added in place of a sample when the PCR reaction is set up.

714 NC: In practice, many laboratories use PBGS or a similar buffer. Ideally the controls
715 for testing of semen samples should be negative semen, from seronegative bulls.
716 However, as a minimum, the assay in use should have been extensively validated
717 with negative and positive samples to confirm that it gives reliable extraction and
718 amplification with semen.

719 PCs: There are two positive controls (PC1=moderate – [Ct 29-32] and PC2=weak
720 [Ct 32–35] positive). Positive semen from naturally infected bulls is preferable as a
721 positive control. However, this is likely to be difficult to obtain. Further, semen from
722 a PI bull is not considered suitable because the virus loads are usually very high
723 and would not give a reliable indication of any moderate reduction in extraction or
724 assay performance. Negative semen spiked with defined quantities of BVDV virus
725 could be used as an alternative. If other samples are used as a routine PC, as a
726 minimum the entire extraction process and PCR assay in use must have been
727 extensively validated using known positive semen from bulls with a PTI or from
728 bulls undergoing an acute infection. If these samples are not available and spiked
729 samples are used for validation purposes, a number of samples spiked with very
730 low levels of virus should be included. On a day-to-day basis, the inclusion of an
731 exogenous control with each test sample will largely compensate for not using
732 positive semen as a control and will give additional benefits by monitoring the
733 efficiency of the assay on each individual sample. Positive control samples should
734 be prepared carefully to avoid cross contamination from high titred virus stocks and
735 should be prepared in advance and frozen at a 'ready to use' concentration and
736 ideally 'single use' volume.

737 f) Extracted samples are added to the PCR mix in a separate room. The controls
738 should be added last, in a consistent sequence in the following order: NTC,
739 negative and then the two positive controls.

740 g) Real-time polymerase chain reaction

741 The PCR plate or tubes are placed in the real-time PCR detection system in a
742 separate, designated PCR room. Some mastermixes have uniform reaction
743 conditions that are suitable for many different assays. As an example, the PCR
744 detection system is programmed for the test as follows:

745	1× 48°C 10 minutes
746	1× 95°C 10 minutes
747	45 × (95°C 15 seconds, 60°C 1 minute)

- 748 h) Analysis of real-time PCR data
749 The software program is usually set to automatically adjust results by
750 compensating for any background signal and the threshold level is usually set
751 according to the manufacturer's instructions for the selected analysis software
752 used. In this instance, a threshold is set at 0.05.
- 753 i) Interpretation of results
- 754 a) Test controls – all controls should give the expected results with positive
755 controls (PC1 and PC2) falling within the designated range and both the
756 negative control (NC) and no template control (NTC) should have no Ct
757 values.
- 758 b) Test samples
- 759 1) Positive result: Any sample that has a cycle threshold (Ct) value less
760 than 40 is regarded as positive.
- 761 2) Negative result: Any sample that shows no Ct value is regarded as
762 negative. However, before reporting a negative result for a sample,
763 the performance of the exogenous internal control should be
764 checked and shown to give a result within the accepted range for
765 that control (for example, a Ct value no more than 2–3 Ct units
766 higher than the NTC).

767 1.3. Enzyme-linked immunosorbent assay for antigen detection

768 Antigen detection by ELISA has become a widely adopted method for the detection of
769 individual PI animals. These assays are not intended for the detection of acutely infected
770 animals (though from time to time this may be achieved). Importantly, these assays are not
771 designed for screening of semen or biological materials used in assays or vaccine
772 manufacture. Several methods for the ELISA for antigen detection have been published and
773 a number of commercial kits are available. Most are based on the sandwich ELISA principle,
774 with a capture antibody bound to the solid phase, and a detector antibody conjugated to a
775 signal system, such as peroxidase. Amplification steps such as the use of biotin and
776 streptavidin in the detection system are sometimes used to increase assay sensitivity. Both
777 monoclonal- and polyclonal-based systems are described. The test measures BVD antigen
778 (NS2-3 or ERNS) in lysates of peripheral blood leukocytes; the new generation of antigen-
779 capture ELISAs (ERNS capture ELISAs) are able to detect BVD antigen in blood as well as in
780 plasma or serum samples. The best of the methods gives a sensitivity similar to virus isolation,
781 and may be preferred in those rare cases where persistent infection is combined with
782 seropositivity. Due to transient viraemia, the antigen ELISA is less useful for virus detection in
783 acute BVD infections.

784 The NS2-3 antigen detection ELISAs may be less effective in young calves that have had
785 colostrum due to the presence of BVDV maternal antibodies, especially when blood samples
786 or blood leucocytes are tested (Fux & Wolf, 2012). Blood or blood leucocytes should not be
787 tested in the first month (ERNS capture ELISA) or the first 3 months (NS2-3 ELISA) of life due
788 to the inhibitory effect of maternal antibodies. The real-time RT-PCR is probably the most
789 sensitive detection method for this circumstance, but the ERNS ELISA has also been shown
790 to be a sensitive and reliable test, particularly when used with skin biopsy (ear-notch) samples
791 (Cornish *et al.*, 2005).

792 1.4. Immunohistochemistry

793 Enzyme-labelled methods are useful to detect BVDV antigen in tissue sections, particularly
794 where suitable MAbs are available. However, these assays are not appropriate to certify
795 animals for international trade and use should be limited to diagnostic investigations. It is
796 important that the reagents and procedures used be fully validated, and that nonspecific
797 reactivity be eliminated. For PI cattle almost any tissue can be used, but particularly good
798 success has been found with lymph nodes, thyroid gland, skin, brain, abomasum and placenta.

799 Skin biopsies, such as ear-notch samples, have shown to be useful for *in-vivo* diagnosis of
800 persistent BVDV infection.

801 2. Serological tests

802 Antibody to BVDV can be detected in cattle sera by a standard VNT or by ELISA, using one of several
803 published methods or with commercial kits (e.g. Edwards, 1990). Serology is used to identify levels of
804 herd immunity, for the detection of the presence of PI animals in a herd, to assist with investigation of
805 reproductive disease and possible involvement of BVDV and to establish the serological status of bulls
806 being used for semen collection and to identify whether there has been a recent infection. ELISA for
807 antibody in bulk milk samples can give a useful indication of the BVD status of a herd (Niskanen, 1993).
808 High ELISA values (0.8 or more absorbance units) in an unvaccinated herd indicates a high probability
809 of the herd having been exposed to BVDV in the recent past, most likely through one or more persistently
810 viraemic animals being present. In contrast, a very low or negative values (≤ 0.2) indicates that it is
811 unlikely that persistently viraemic animals are present. However, ELISA values are not always a reliable
812 indicator of the presence of PI animals on farms, due to differing husbandry (Zimmer *et al.*, 2002), recent
813 administration of vaccine and also due to the presence of viral antigen in bulk milk, which may interfere
814 with the antibody assay itself. Determination of the antibody status of a small number of young stock
815 (9–18 months) has also been utilised as an indicator of recent transmission of BVDV in the herd (Houe
816 *et al.*, 1995), but this approach is also dependent on the degree of contact between different groups of
817 animals in the herd and the potential for exposure from neighbouring herds. VN tests are more frequently
818 used for regulatory purposes (e.g. testing of semen donors) while ELISAs (usually in the form of
819 commercially prepared kits) are commonly used for diagnostic applications. Whether ELISA or VNT,
820 control positive and negative standard sera must be included in every test. These should give results
821 within predetermined limits for the test to be considered valid. In the VNT, a 'serum control' to monitor
822 sample toxicity should also be included for each test sample.

823 2.1. Virus neutralisation test

824 Selection of the virus strain to include in a VNT is very important. No single strain is likely to
825 be ideal for all circumstances, but in practice one should be selected that detects the highest
826 proportion of serological reactions in the local cattle population. Low levels of antibody to
827 BVDV type 2 virus (*Pestivirus tauri*) may not be detectable by a neutralisation test that uses
828 type 1 strain of the virus, and vice versa (Fulton *et al.*, 1997). It is important that BVDV type 1
829 and BVDV type 2 (*Pestivirus bovis* and *P. tauri*) be used in the test and not just the one that
830 the diagnostician thinks is present, as this can lead to under reporting. Because it makes the
831 test easier to read, most laboratories use highly cytopathic, laboratory-adapted strains of
832 BVDV for VN tests. Two widely used cytopathic strains are 'Oregon C24V' and 'NADL'.
833 However immune-labelling techniques are now available that allow simple detection of the
834 growth or neutralisation of non-cytopathic strains where this is considered desirable, especially
835 to support the inclusion of a locally relevant virus strain. An outline protocol for a microtitre VN
836 test is given below (Edwards, 1990):

837 2.1.1. Test procedure

- 838 i) The test sera are heat-inactivated for 30 minutes at 56°C.
- 839 ii) From a starting dilution of 1/4, serial twofold dilutions of the test sera are made in
840 a cell-culture grade flat-bottomed 96-well microtitre plate, using cell culture medium
841 as diluent. For each sample, three or four wells are used at each dilution depending
842 on the degree of precision required. At each dilution of serum, for each sample one
843 well is left without virus to monitor for evidence of sample toxicity that could mimic
844 viral cytopathology or interfere with virus replication. Control positive and negative
845 sera should also be included in each batch of tests.
- 846 iii) An equal volume (e.g. 50 μ l) of a stock of cytopathic strain of BVDV containing 100
847 TCID₅₀ (50% tissue culture infective dose) is added to each well. A back titration of
848 virus stock is also done in some spare wells to check the potency of the virus
849 (acceptance limits 30–300 TCID₅₀).
- 850 iv) The plate is incubated for 1 hour at 37°C.

- 851 v) A flask of suitable cells (e.g. bovine turbinate, bovine testis) is trypsinised and the
852 cell concentration is adjusted to 1.5×10^5 /ml. 100 μ l of the cell suspension is added
853 to each well of the microtitre plate.
- 854 vi) The plate is incubated at 37°C for 4–5 days, either in a 5% CO₂ atmosphere or with
855 the plate sealed.
- 856 vii) The wells are examined microscopically for CPE or fixed and stained by
857 immunoperoxidase staining using an appropriate monoclonal antibody. The VN
858 titre for each serum is the dilution at which the virus is neutralised in 50% of the
859 wells. This can be calculated by the Spearman–Kärber or Reed Muench methods.
860 A seronegative animal will show no neutralisation at the lowest dilution (1/4),
861 equivalent to a final dilution of 1/8. For accurate comparison of antibody titres, and
862 particularly to demonstrate significant (more than fourfold) changes in titre,
863 samples should be tested in parallel in the same test.

864 2.2. Enzyme-linked immunosorbent assay

865 Both indirect and blocking types of test can be used. A number of commercial kits are
866 available. As with the virus neutralisation test, ELISAs configured using antigen from one
867 genotype-species of BVDV may not efficiently detect antibody induced by another genotype
868 virus species. Tests should therefore be selected for their ability to detect antibody to the
869 spectrum of types and strains circulating in the country where the test is to be performed.

870 The chief difficulty in setting up the test lies in the preparation of a viral antigen of sufficient
871 potency. The virus must be grown under optimal culture conditions using a highly permissive
872 cell type. Any serum used in the medium must not inhibit growth of BVDV. The optimal time
873 for harvest should be determined experimentally for the individual culture system. The virus
874 can be concentrated and purified by density gradient centrifugation. Alternatively, a potent
875 antigen can be prepared by treatment of infected cell cultures with detergents, such as Nonidet
876 P40, N-decanoyl-N-methylglucamine (Mega 10), Triton X-100 or 1-octylbeta-D-
877 glucopyranoside (OGP). Some workers have used fixed, infected whole cells as antigen. ~~In~~
878 ~~the future,~~ Increasing use ~~may be is~~ made of artificial antigens manufactured by expressing
879 specific viral genes in bacterial or eukaryotic systems. Such systems should be validated by
880 testing sera specific to a wide range of different virus strains. In the future, this technology
881 should enable the production of serological tests complementary to subunit or marker
882 vaccines, thus enabling differentiation between vaccinated and naturally infected cattle. An
883 example outline protocol for an indirect ELISA is given below (Edwards, 1990).

884 2.2.1. Test procedure

- 885 i) Roller cultures of secondary calf testis cells with a high multiplicity of infection
886 (about one), are inoculated with BVDV strain Oregon C24V, overlaid with serum-
887 free medium and incubated for 24 hours at 37°C.
- 888 ii) The cells are scraped off and pelleted. The supernatant medium is discarded. The
889 pellet is treated with two volumes of 2% OGP in PBS for 15 minutes at 4°C, and
890 centrifuged to remove the cell debris. The supernatant antigen is stored in small
891 aliquots at –70°C, or freeze-dried. Non-infected cells are processed in parallel to
892 make a control antigen.
- 893 iii) The antigen is diluted to a predetermined dilution in 0.05 M bicarbonate buffer, pH
894 9.6. Alternate rows of an ELISA-grade microtitre plate are coated with virus and
895 control antigens overnight at 4°C. The plates are then washed in PBS with 0.05%
896 Tween 20 or Tween 80 (PBST) before use in the test.
- 897 iv) Test sera are diluted 1/50 in serum diluent (0.5 M NaCl; 0.01 M phosphate buffer;
898 0.05% Tween 20; 0.001 M ethylene diamine tetra-acetic acid; 1% polyvinyl
899 pyrrolidone, pH 7.2) and added to virus- and control-coated wells for 1 hour at
900 37°C. The plates are then washed five times in PBST.
- 901 v) Rabbit anti-bovine IgG peroxidase conjugate is added at a predetermined dilution
902 (in serum diluent) for 1 hour at 37°C, then the plates are again washed five times
903 in PBST.

- 904 vi) A suitable enzyme substrate is added, such as hydrogen peroxide/tetramethyl
905 benzidine. After colour development, the reaction is stopped with sulphuric acid
906 and the absorbance is read on an ELISA plate reader. The value obtained with
907 control antigen is subtracted from the test reaction to give a net absorbance value
908 for each serum.
- 909 vii) It is recommended to convert net absorbance values to sample:positive ratio (or
910 percentage positivity) by dividing net absorbance by the net absorbance on that
911 test of a standard positive serum that has a net absorbance of about 1.0. This
912 normalisation procedure leads to more consistent and reproducible results.

913 C. REQUIREMENTS FOR VACCINES

914 1. Background

915 BVDV vaccines are used primarily for disease control purposes. Although they can convey production
916 advantages especially in intensively managed cattle such as in feedlots. In some countries where BVDV
917 eradication is being undertaken, PI animals are removed and remaining cattle are vaccinated to maintain
918 a high level of ~~infection-antibody positivity~~ and prevent the generation of further PI animals. Vaccination
919 to control BVDV infections can be challenging due in part to the antigenic variability of the virus and the
920 occurrence of persistent infections that arise as a result of fetal infection. Ongoing maintenance of the
921 virus in nature is predominantly sustained by PI animals that are the product of *in-utero* infection. The
922 goal for a vaccine should be to prevent systemic viraemia and the virus crossing the placenta. If this is
923 successfully achieved it is likely that the vaccine will prevent the wide range of other clinical
924 manifestations, including reproductive, respiratory and enteric diseases and immunosuppression with
925 its secondary sequelae. There are many different vaccines available in different countries. Traditionally,
926 BVD vaccines fall into two classes: modified live virus or inactivated vaccines. Experimental recombinant
927 subunit vaccines based on BVD viral glycoprotein E2 expressed with baculovirus ~~or~~ transgenic plants
928 or heterologous viruses and BVDV E2 DNA vaccines have been described but few, if any, are in
929 commercial production. They offer a future prospect of 'marker vaccines' when used in connection with
930 a complementary serological test.

931 1.1. Characteristics of a target product profile

932 Traditionally, BVD vaccines fall into two classes: modified live or inactivated virus vaccines.
933 The essential requirement for both types is to ~~afford~~ provide a high level of fetal ~~infection~~
934 protection. Many of the live vaccines have been based on a cytopathic strain of the virus which
935 is considered to be unable to cross the placenta. However, it is important to ensure that the
936 vaccine virus does not cause fetal infection. In general, vaccination of breeding animals should
937 be completed well before insemination to ensure optimal protection and avoid any risk of fetal
938 infection. Live virus vaccine may also be immunosuppressive and precipitate other infections.
939 On the other hand, modified live virus vaccines may only require a single dose. Use of a live
940 product containing a cytopathic strain of BVDV may precipitate mucosal disease by
941 superinfection of persistently viraemic animals. Properly formulated inactivated vaccines are
942 very safe to use but, to obtain satisfactory levels of immunity, they usually require booster
943 vaccinations, which may be inconvenient. A combined vaccination protocol using inactivated
944 followed by live vaccine may reduce the risk of adverse reaction to the live strain. Whether live
945 or inactivated, because of the propensity for antigenic variability, the vaccine should contain
946 strains of BVDV that are closely matched to viruses found in the area in which they are used.
947 For example, in countries where strains of BVDV type 2 (*Pestivirus tauri*) are found, it is
948 important for the vaccine to contain a suitable type 2 strain. For optimal immunity against type
949 1 strains (*Pestivirus bovis*), antigens from the dominant subtypes (e.g. 1a and 1b) should be
950 included. Due to the need to customise vaccines for the most commonly encountered strains
951 within a country or region, it is not feasible to produce a vaccine antigen bank that can be
952 drawn upon globally.

953 Guidance for the production of veterinary vaccines is given in Chapter 1.1.8 *Principles of*
954 *veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to
955 be general in nature and may be supplemented by national and regional requirements.

956 2. Outline of production and minimum requirements for vaccines

957 2.1. Characteristics of the seed

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

964 2.1.1. Biological characteristics of the master seed

965 Isolates of cytopathic virus are often mixed with the noncytopathic biotype. The
966 separation and purification of the two biotypes from an initial mixed culture is important
967 to maintain the expected characteristics of the ~~seed~~ seed and depends on several
968 cycles of a limiting dilution technique for the noncytopathic virus, or plaque selection for
969 the cytopathic virus. Purity of the cytopathic virus should be confirmed by at least one
970 additional passage at limiting dilution. When isolates have been cloned, their identity
971 and key antigenic characteristics should be confirmed. The identity of the seed virus
972 should be confirmed by sequencing. Where there are multiple isolates included in the
973 vaccine, each has to be prepared separately.

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

987 2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

988 It is crucial to ensure that all materials used in the preparation of the bulk antigens have
989 been extensively screened to ensure freedom from extraneous agents. This should
990 include master and working seeds, the cell cultures and all medium supplements such
991 as bovine serum. It is particularly important to ensure that any serum used that is of
992 bovine origin is free of both adventitious BVDV of all ~~genotypes~~ and antibodies against
993 BVDV strains because low levels of either virus or antibody can mask the presence of
994 the other. Materials and vaccine seeds should be tested for sterility and freedom from
995 contamination with other agents, especially viruses as described in the chapter 1.1.8
996 and chapter 1.1.9.

997 2.1.3. Validation as a vaccine strain

998 All vaccines should pass standard tests for efficacy. Tests should include as a minimum
999 the demonstration of a neutralising antibody response following vaccination, a reduction
1000 in virus shedding after challenge in vaccinated cattle and ideally a prevention of
1001 viraemia. Efficacy tests of BVD vaccines by assessing clinical parameters in non-
1002 pregnant cattle can be limited by the difficulty of consistently establishing clinical signs
1003 but, when employed, clinical parameters such as a reduction in the rectal temperature
1004 response and leukopenia should be monitored. Although it can be difficult by using virus
1005 isolation in cell culture to consistently demonstrate the low levels of viraemia associated
1006 with an acute infection, real-time PCR could be considered as an alternative method to
1007 establish the levels of circulating virus.

1008 If a vaccine passes basic tests, the efficacy of vaccination should ultimately be
1009 measured by the capacity to prevent transplacental transmission. If there is a substantial
1010 reduction and ideally complete prevention of fetal infection, a vaccine would be
1011 expected to be highly effective in other situations (for example prevention of respiratory
1012 disease). A suitable challenge system can be established by intranasal inoculation of
1013 noncytopathic virus into pregnant cows between 60 and 90 days of gestation (Brownlie
1014 *et al.*, 1995). Usually this system will reliably produce persistently viraemic offspring in
1015 non-immune cows. In countries where BVDV type 2 viruses (*Pestivirus tauri*) are
1016 commonly encountered, efficacy in protecting against BVDV type 2 infections should be
1017 measured.

1018 **2.2. Method of manufacture**

1019 **2.2.1. Procedure**

1020 Both cytopathic and noncytopathic biotypes will grow in a variety of cell cultures of
1021 bovine origin. Standard procedures may be used, with the expectation for harvesting
1022 noncytopathic virus on days 4–7 and cytopathic virus on days 2–4. The optimal yield of
1023 infectious virus will depend on several factors, including the cell culture, isolate used
1024 and the initial seeding rate of virus. These factors should be taken into consideration
1025 and virus replication kinetics investigated to establish the optimal conditions for large
1026 scale virus production. Whether a live or inactivated vaccine, the essential aim will be
1027 to produce a high-titred virus stock. This bulk antigen preparation can subsequently be
1028 prepared according to the type of vaccine being considered.

1029 **2.2.2. Requirements for ingredients**

1030 Most BVDV vaccines are grown in cell cultures of bovine origin that are frequently
1031 supplemented with medium components of animal origin. The material of greatest
1032 concern is bovine serum due to the potential for contamination with BVD viruses and
1033 antibodies to these viruses. These adventitious contaminants not only affect the
1034 efficiency of production but also may mask the presence of low levels of infectious
1035 BVDV that may have undesirable characteristics. In addition to the virus seeds, all
1036 materials should be tested for sterility and freedom from contamination with other
1037 agents, especially viruses as described in chapters 1.1.8 and 1.1.9. Further, materials
1038 of bovine or ovine origin should originate from a country with negligible risk for
1039 transmissible spongiform encephalopathies [TSEs] (see chapter 1.1.9).

1040 **2.2.3. In-process controls**

1041 In-process controls are part of the manufacturing process. Cultures should be inspected
1042 regularly to ensure that they remain free from contamination, and to monitor the health
1043 of the cells and the development or absence of CPE, as appropriate. While the basic
1044 requirement for efficacy is the capacity to induce an acceptable neutralising antibody
1045 response, during production, target concentrations of antigen required to achieve an
1046 acceptable response may be monitored indirectly by assessment of the quantity of
1047 infectious virus or antigen mass that is produced. Rapid diagnostic assays such as the
1048 ELISA are useful to monitor BVDV antigen production. Alternatively, the quality of a
1049 batch of antigen may be determined by titration of the quantity of infectious virus
1050 present, although this may underestimate the quantity of antigen. For inactivated
1051 vaccines, infectivity is evaluated before inactivation. For inactivated vaccines the
1052 inactivation kinetics should be established so that a suitable safety margin can be
1053 determined and incorporated into the routine production processes. At the end of
1054 production, *in-vitro* cell culture assays should be undertaken to confirm that inactivation
1055 has been complete. These innocuity tests should include a sufficient number of
1056 passages and volume of inoculum to ensure that very low levels of infectious virus would
1057 be detected if present.

- 1058 **2.2.4. Final product batch tests**
- 1059 i) Sterility
- 1060 Tests for sterility and freedom from contamination of biological materials intended
- 1061 for veterinary use may be found in Chapter 1.1.9.
- 1062 ii) Identity
- 1063 Identity tests should demonstrate that no other strain of BVDV is present when
- 1064 several strains are propagated in a facility producing multivalent vaccines.
- 1065 iii) Safety
- 1066 Safety tests shall consist of detecting any abnormal local or systemic adverse
- 1067 reactions to the vaccine by all vaccination route(s). Batch-to-batch safety tests are
- 1068 required unless safety of the product is demonstrated and APPROVED in the
- 1069 registration dossier and production is consistent with that described in chapter
- 1070 1.1.8.
- 1071 The safety test is different to the innocuity test (see above).
- 1072 Live vaccines must either be demonstrated to be safe in pregnant cattle (i.e. no
- 1073 transmission to the fetus), or should be licensed with a warning not to use them in
- 1074 pregnant animals. Live vaccines containing cytopathic strains should have an
- 1075 appropriate warning of the risk of inducing mucosal disease in PI cattle.
- 1076 iv) Batch potency
- 1077 BVD vaccines must be demonstrated to produce adequate immune responses,
- 1078 when used in their final formulation according to the manufacturer's published
- 1079 instructions. The minimum quantity of infectious virus and/or antigen required to
- 1080 produce an acceptable immune response should be determined. *In-vitro* assays
- 1081 should be used to monitor individual batches during production.

1082 **2.3. Requirements for authorisation/registration/licensing**

1083 **2.3.1. Manufacturing process**

1084 For registration of a vaccine, all relevant details concerning manufacture of the vaccine

1085 and quality control testing should be submitted to the relevant authorities. Unless

1086 otherwise specified by the authorities, information should be provided from three

1087 consecutive vaccine batches with a volume not less than 1/3 of the typical industrial

1088 batch volume.

1089 There is no standard method for the manufacture of a BVD vaccine, but conventional

1090 laboratory techniques with stationary, rolled or suspension (micro-carriers) cell cultures

1091 may be used. Inactivated vaccines can be prepared by conventional methods, such as

1092 binary ethylenimine or beta-propiolactone inactivation (Park & Bolin, 1987). A variety of

1093 adjuvants may be used.

1094 **2.3.2. Safety requirements**

1095 *In-vivo* tests should be undertaken using a single dose, overdose (for live vaccines only)

1096 and repeat doses (taking into account the maximum number of doses for primary

1097 vaccination and, if appropriate, the first revaccination/booster vaccination) and contain

1098 the maximum permitted antigen load and, depending on the formulation of the vaccine,

1099 the maximum number of vaccine strains.

1100 i) Target and non-target animal safety

1101 The safety of the final product formulation of both live and inactivated vaccines

1102 should be assessed in susceptible young calves that are free of maternally derived

1103 antibodies and in pregnant cattle. They should be checked for any local reactions

1104 following administration, and, in pregnant cattle, for any effects on the unborn calf.

1105 Live attenuated vaccines may contribute to immunosuppression that might
1106 increase mortality. It may also contribute to the development of mucosal disease
1107 in PI animals that is an animal welfare concern. Therefore vaccination of PI animals
1108 with live attenuated vaccines containing cytopathic BVDV should be avoided. Live
1109 attenuated vaccines must not be capable of being transmitted to other
1110 unvaccinated animals that are in close contact.

1111 ii) Reversion-to-virulence for attenuated/live vaccines and environmental
1112 considerations

1113 Virus seeds that have been passaged at least up to and preferably beyond the
1114 passage limit specified for the seed should be inoculated into young calves to
1115 confirm that there is no evidence of disease. If a live attenuated vaccine has been
1116 registered for use in pregnant animals, reversion to virulence tests should also
1117 include pregnant animals. Live attenuated vaccines should not be transmissible to
1118 unvaccinated 'in-contact' animals.

1119 iii) Precautions (hazards)

1120 BVDV is not considered to be a human health hazard. Standard good
1121 microbiological practice should be adequate for handling the virus in the laboratory.
1122 A live virus vaccine should be identified as harmless for people administering the
1123 product. However adjuvants included in either live or inactivated vaccines may
1124 cause injury to people. Manufacturers should provide adequate warnings that
1125 medical advice should be sought in the case of self-injection (including for
1126 adjuvants, oil-emulsion vaccine, preservatives, etc.) with warnings included on the
1127 product label/leaflet so that the vaccinator is aware of any danger.

1128 **2.3.3. Efficacy requirements**

1129 The potency of the vaccine should be determined by inoculation into seronegative and
1130 virus negative calves, followed by monitoring of the antibody response. Antigen content
1131 can be assayed by ELISA and adjusted as required to a standard level for the particular
1132 vaccine. Standardised assay protocols applicable to all vaccines do not exist. Live
1133 vaccine batches may be assayed by infectivity titration. Each production batch of
1134 vaccine should undergo potency and safety testing as batch release criteria. BVD
1135 vaccines must be demonstrated to produce adequate immune responses, as outlined
1136 above, when used in their final formulation according to the manufacturer's published
1137 instructions.

1138 **2.3.4. Vaccines permitting a DIVA strategy (detection of infection in vaccinated animals)**

1139 To date, there are no commercially available vaccines for BVDV that support use of a
1140 true DIVA strategy. Experimental subunit vaccines based on baculovirus-expressed
1141 BVD viral glycoprotein E2 have been described but are not available commercially. They
1142 offer a future prospect of 'marker vaccines' when used in connection with a
1143 complementary serological test. Experimental BVDV E2 DNA vaccines and BVDV E2
1144 subunit vaccines expressed using transgenic plants and alphavirus replicon or chimeric
1145 pestivirus vaccines have also been described.

1146 **2.3.5. Duration of immunity**

1147 There are few published data on the duration of antibody following vaccination with a
1148 commercial product. Protocols for their use usually recommend a primary course of two
1149 inoculations and boosters at yearly intervals. Only limited data are available on the
1150 antibody levels that correlate with protection against respiratory infections (Bolin &
1151 Ridpath, 1995; Howard *et al.*, 1989) or *in-utero* infection (Brownlie *et al.*, 1995).
1152 However, there are many different commercial formulations and these involve a range
1153 of adjuvants that may support different periods of efficacy. Consequently, duration of
1154 immunity data must be generated separately for each commercially available product
1155 by undertaking challenge tests at the end of the period for which immunity has been
1156 claimed.

1157 **2.3.6. Stability**

1158 There are no accepted guidelines for the stability of BVD vaccines, but it can be
1159 assumed that attenuated virus vaccine (freeze-dried) should remain potent for at least
1160 1 year if kept at 4°C. Inactivated virus vaccine could have a longer shelf life at 4°C.
1161 Lower temperatures could prolong shelf life for either type, but adjuvants in killed
1162 vaccine may preclude this. Bulk antigens that have not been formulated into finished
1163 vaccine can be reliably stored frozen at low temperatures but the antigen quality should
1164 be monitored with *in-vitro* assays prior to incorporation into a batch of vaccine.

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- 1256 *
 1257 * *
- 1258 **NB:** There are WOAHO Reference Laboratories for bovine viral diarrhoea (please consult the WOAHO
 1259 Web site:
 1260 <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>)
 1261 Please contact the WOAHO Reference Laboratories for any further information on
 1262 diagnostic tests, reagents and vaccines for bovine viral diarrhoea
- 1263 **NB:** FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2015.

1
2

Appendix 1: Bovine viral diarrhoea
Intended purpose of test: population freedom from infection

Test with score and species	Sample type and target analytes	Accuracy	Test population used to measure accuracy	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
NA detection by (real-time) RT-PCR +++	Ear notch (skin), blood, milk	Performance has been demonstrated under field conditions in large control programs	Whole Swiss, German and Irish cattle populations	See references	<ul style="list-style-type: none"> - Very sensitive - Rapid - High-throughput - Well established internationally - Detects assay-dependent all BVDV species - Allows assay-dependent for differentiation of BVDV types 1 and 2 - Detects persistent and transient infection - Proficiency panel of different Pestivirus strains available - Detection of viral RNA in skin biopsy samples unaffected by maternally-derived antibodies, therefore allows for identification of persistently infected animals early in life - Successfully applied in ongoing or completed control programmes 	<ul style="list-style-type: none"> - Possibility for contamination at sample collection or in laboratory, leading to false positive results - Needs specialised equipment - Detection of viral RNA does not imply per se that infectious virus is present 	<ul style="list-style-type: none"> - Presi & Heim (2010). <i>Vet. Microbiol.</i>, 142, 137–142 - Schweizer <i>et al.</i> (2021) <i>Front. Vet. Sci.</i>, 8, 702730 - Wernike <i>et al.</i> (2017). <i>Pathogens</i>, 6 (4) - Graham <i>et al.</i> (2021) <i>Front. Vet. Sci.</i>, 8, 674557
Antibody detection by ELISA +++	Bulk milk, blood	DSe and DSp differs based on the ELISA used (commercial/in-house) and the antibodies being tested (e.g. antibodies against structural (E2) and non-structural (NS2-3) proteins.			<ul style="list-style-type: none"> - Simple to perform and cost-effective - Milk collection is non-invasive method with potential for herd screening with tank/bulk milk samples - Bulk milk sensitive indicator for PI in herd 	<ul style="list-style-type: none"> - Some cross-reactivity with vaccines and other pestiviruses - PI animal will usually be seronegative - Bulk milk from herd excludes males, non-lactating or young stock 	<ul style="list-style-type: none"> Beaudeau <i>et al.</i> (2001). <i>Vet. Microbiol.</i>, 80, 329–337 Lanyon <i>et al.</i> (2013). <i>Aust. Vet. J.</i>, 91, 52–56.

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population used to measure accuracy</u>	<u>Validation report</u>	<u>Advantages: expert opinion</u>	<u>Disadvantages: expert opinion</u>	<u>References</u>
<u>Antigen detection by ELISA +++</u>	<u>Serum, whole blood, skin biopsy</u>	<u>DSe 67–100% and DSp 98.8–100% relative to virus isolation reported</u>			<u>Relatively simple to perform, rapid, can be cost-effective (when compared to virus isolation and PCR) and suitable for high-throughput applications. There is no need for cell culture facility.</u>	<u>Maternal antibodies in colostrum may interfere with testing for antigen in serum using ELISA in calves. Ear notch samples are less affected. PI calves <i>in utero</i> defies detection.</u>	<u>Lanyon <i>et al.</i> (2013). <i>Vet. J.</i> 199, 201–209;</u>
<u>Virus isolation +</u>	<u>Serum, whole blood</u>	<u>Considered (historically) reference test: DSe <90% compared with real-time RT-PCR ; DSp ~100%</u>	<u>N/A</u>	<u>Historical information with no formal validation</u>	<u>- High degree of specificity - Identifies presence of infectious virus</u>	<u>- Requires specialised cell culture capabilities and access to BVDV free materials - Reduced sensitivity in presence of maternally-derived antibodies</u>	<u>N/A</u>
<u>Virus neutralisation test +</u>	<u>Serum</u>	<u>DSe & DSp both extremely high, both >99%. Historical reference serological test.</u>	<u>N/A</u>	<u>Historical information with no formal validation</u>	<u>Very high specificity</u>	<u>- ASe can vary depending on virus strain used - Requires cell culture, good quality samples - Labour intensive, takes 5 days to obtain results - Expensive</u>	<u>N/A</u>

3 N/A: not available

Appendix 2: Bovine viral diarrhoea

Intended purpose of test: individual animal freedom from infection prior to movement

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population used to measure accuracy</u>	<u>Validation Report</u>	<u>Advantages: expert opinion</u>	<u>Disadvantages: expert opinion</u>	<u>References</u>
<u>Virus isolation ++</u>	<u>Serum, whole blood.</u>	<u>Considered reference test; DSe <90% compared with real-time RT-PCR; DSp ~100%</u>	<u>N/A</u>	<u>Historical information with no formal validation</u>	<ul style="list-style-type: none"> - <u>High degree of specificity</u> - <u>Identifies presence of infectious virus</u> 	<ul style="list-style-type: none"> - <u>Requires specialised cell culture capabilities and access to BVDV free materials</u> - <u>Reduced sensitivity in presence of MDA (diagnostic gap); takes several weeks for maximum DSe</u> 	<u>Edmonson et al. (2007); Toker & Yesilbag (2021)</u>
<u>Antigen detection by ELISA +++</u>	<u>Serum, whole blood, skin biopsy (e.g. ear notch)</u>	<u>DSe 67–100% and DSp 98.8–100% relative to virus isolation have been reported but usually DSe and DSp are extremely high</u>			<u>Relatively simple to perform, rapid, can be cost-effective and suitable for high-throughput applications. There is no need for cell culture facility.</u>	<u>Maternal antibodies in colostrum may interfere with testing for antigen in serum using ELISA in calves. Ear notch samples are less affected. PI calves <i>in utero</i> defies detection.</u>	<u>Zimmer et al. (2004). <i>Vet. Microbiol.</i> 100, 145–149.</u>
<u>NA detection by (real-time) RT-PCR +++</u>	<u>Ear notch (skin), blood; nasal or oral swab</u>	<ul style="list-style-type: none"> - <u>Depending on the assay analytical sensitivity of less than 10 genome copies/reaction</u> - <u>Accuracy 98.73% based on a proficiency test panel consisting of five ear notch and five serum samples</u> 		<u>See references</u>	<ul style="list-style-type: none"> - <u>Very sensitive</u> - <u>Rapid</u> - <u>High-throughput</u> - <u>Well established internationally</u> - <u>Depending on the assay detects all BVDV species</u> - <u>Allows assay-dependent for differentiation of BVDV types 1 and 2</u> - <u>Detects persistent and transient infection</u> - <u>Proficiency panel of different Pestivirus strains available</u> - <u>Detection of viral RNA in skin biopsy samples unaffected by maternally-derived antibodies</u> 	<ul style="list-style-type: none"> - <u>Possibility for contamination at sample collection or in laboratory, leading to false positive results</u> - <u>Needs specialised equipment</u> 	<ul style="list-style-type: none"> - <u>Hoffmann et al. (2006). <i>J. Virol. Methods</i>, 136, 200–209.</u> - <u>Wernike et al. (2019). <i>Vet. Microbiol.</i>, 239, 108452.</u>

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population used to measure accuracy</u>	<u>Validation Report</u>	<u>Advantages: expert opinion</u>	<u>Disadvantages: expert opinion</u>	<u>References</u>
<u>Virus neutralisation test ++</u>	<u>Serum</u>	<u>DSe & DSp both extremely high, both >99%. Historical reference serological test.</u>	<u>N/A</u>	<u>Historical information with no formal validation</u>	<p><u>Very high specificity</u></p> <ul style="list-style-type: none"> - <u>Negative results generally indicate that an animal has not been infected provided it has been tested for virus/antigen</u> <p><u>Animals that give positive results have usually cleared the infection provided that they are not pregnant. Bulls present a special situation. If they seroconvert during a period of quarantine, there is a risk that there will be virus in their semen. Further, a small proportion of seropositive bulls may have a persistent testicular infection that may last for many months. To ensure freedom from infection, semen must be tested on several occasions using real-time RT-PCR. Virus isolation is not sufficiently sensitive and the presence of virus may be masked by antibodies.</u></p>	<ul style="list-style-type: none"> - <u>ASe can vary depending on virus strain used</u> - <u>Requires cell culture, good quality samples</u> - <u>time consuming to perform, takes 5 days to obtain results</u> - <u>Labour intensive</u> - <u>Due to the biology of the virus (birth of PI calves that are not able to amount a specific immune response to the virus strain they are infected with) antibody-negative animals could be PI (in non-BVDV-free populations)</u> 	<u>N/A</u>
<u>Antibody detection by ELISA ++</u>	<u>Blood, Individual milk sample</u>	<u>DSe and DSp differs based on the ELISA used (commercial/in-house) and the antibodies being tested (e.g. antibodies against structural (E2) and non-structural (NS2-3) proteins.</u>			<ul style="list-style-type: none"> - <u>Simple to perform and cost-effective</u> - <u>Milk collection is non-invasive method</u> - <u>Paired samples can be used to confirm acute infection.</u> - <u>Ensure milk is collected directly from the teat rather than bulk-milk tank to ensure no cross contamination/false-positives</u> 	<ul style="list-style-type: none"> - <u>Maternal antibodies in colostrum may interfere with testing for antibodies in serum using ELISA in calves. Calves should be tested after 9 months of age after maternal antibodies have waned.</u> - <u>PI animal will be seronegative and may impact receiving herds if moved.</u> - <u>Using milk, limited to lactating cow only</u> 	<u>N/A</u>

6 N/A: not available

Appendix 3: Bovine viral diarrhoea
Intended purpose of test: contribute to eradication policies

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population used to measure accuracy</u>	<u>Validation report</u>	<u>Advantages: expert opinion</u>	<u>Disadvantages: expert opinion</u>	<u>References</u>
<u>Antigen detection by ELISA +++</u>	<u>Serum, whole blood, skin biopsy</u>	<u>DSse 67%–100% and DSp 98.8–100% reported</u>			<u>Relatively simple to perform, rapid, can be cost-effective and suitable for high-throughput applications. There is no need for cell culture facility.</u>	<u>Maternal antibodies in colostrum may interfere with testing for antigen in serum using ELISA in calves. Ear notch samples are less affected. PI calves <i>in utero</i> defy detection.</u>	<u>Zimmer <i>et al.</i> (2004). <i>Vet. Microbiol.</i>, 100, 145–149</u>
<u>NA detection by (real-time) RT-PCR +++</u>	<u>Ear notch (skin), blood; milk; nasal or oral swab</u>	<u>Utility has been demonstrated under field conditions in large control programs</u>	<u>Whole Swiss, German and Irish cattle populations</u>	<u>See references</u>	<ul style="list-style-type: none"> - <u>Very sensitive</u> - <u>Rapid</u> - <u>High-throughput</u> - <u>Well established internationally</u> - <u>Depending on assay, detects all BVDV species</u> - <u>Allows assay-dependent differentiation of BVDV types 1 and 2</u> - <u>Detects persistent and transient infection</u> - <u>Proficiency panel of different Pestivirus strains available</u> - <u>Detection of viral RNA in skin biopsy samples unaffected from maternally-derived antibodies, therefore allows for identification of persistently infected animals early in life</u> - <u>Successfully applied in ongoing or completed control programmes (see references)</u> 	<ul style="list-style-type: none"> - <u>Possibility for contamination at sample collection or in laboratory, leading to false positive results</u> - <u>Needs specialised equipment</u> 	<ul style="list-style-type: none"> - <u>Presi & Heim (2010). <i>Vet. Microbiol.</i>, 142, 137–142</u> - <u>Schweizer <i>et al.</i> (2021). <i>Front. Vet. Sci.</i>, 8, 702730</u> - <u>Wernike <i>et al.</i> (2017). <i>Pathogens</i>, 6 (4)</u> - <u>Graham <i>et al.</i> (2021). <i>Front. Vet. Sci.</i>, 8, 674557</u>

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population used to measure accuracy</u>	<u>Validation report</u>	<u>Advantages: expert opinion</u>	<u>Disadvantages: expert opinion</u>	<u>References</u>
<u>Antibody detection by ELISA ++</u>	<u>Bulk milk, Blood</u>	<u>DSe and DSp differs based on the ELISA used (commercial/in-house) and the antibodies being tested (e.g. antibodies against structural (E2) and non-structural (NS2-3) proteins.</u>			<ul style="list-style-type: none"> - Simple to perform and cost-effective - Milk collection is non-invasive method - Can be used on a population basis to monitor freedom from infection by selection of appropriate aged animals e.g. borne after eradication achieved; for dairy herds by testing tank milk to monitor lactating animals 	<ul style="list-style-type: none"> - Some cross-reactivity with antibodies from vaccines and other pestiviruses - PI animal will be seronegative - Bulk milk from herd does not include males, non-lactating or young stock 	<u>Laureyns et al. (2010)</u>
<u>Virus isolation ++</u>	<u>Serum, whole blood</u>	<u>Considered reference test ; DSe <90% compared with real-time RT-PCR; DSp ~100%</u>	<u>N/A</u>	<u>Historical information with no formal validation</u>	<ul style="list-style-type: none"> - High degree of specificity - Identifies presence of infectious virus. - used to confirm the status of difficult cases and to provide isolates for intensive analysis e.g. NA sequencing 	<ul style="list-style-type: none"> - Requires specialised cell culture capabilities and access to BVDV free materials - Reduced sensitivity in presence of MDA (diagnostic gap) 	<u>N/A</u>
<u>Virus neutralisation test ++</u>	<u>Serum</u>	<u>DSe & DSp both extremely high, both >99%. Historical reference serological test.</u>	<u>N/A</u>	<u>Historical information with no formal validation</u>	<ul style="list-style-type: none"> <u>Very high specificity</u> - Used for confirming the virus free status of a population after eradication; - Used as a confirmatory test when surveillance utilises an ELISA 	<ul style="list-style-type: none"> - ASe can vary depending on virus strain used - Requires cell culture, good quality samples - Takes 5 days to obtain results 	<u>N/A</u>

9 N/A: not available

Appendix 4: Bovine viral diarrhoea
Intended purpose of test: confirmation of clinical cases

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population used to measure accuracy</u>	<u>Validation report</u>	<u>Advantages: expert opinion</u>	<u>Disadvantages: expert opinion</u>	<u>References</u>
<u>Virus isolation ++</u>	<u>Serum, whole blood, tissue extracts</u>	<u>Considered reference test; DSe <90% compared with real-time RT-PCR; DSp ~100%</u>	<u>Not available</u>	<u>Historical information with no formal validation</u>	<u>- High degree of specificity - Identifies presence of infectious virus - Preferred method to identify presence of cytopathogenic strains and hence confirmation of mucosal disease - Provides virus isolates for detailed characterisation</u>	<u>- Requires specialised cell culture capabilities and access to BVDV free materials - Reduced sensitivity in presence of maternally derived antibodies (diagnostic gap) - Requires high quality samples to avoid bacterial contamination</u>	<u>- Meyling (1984)</u>
<u>Antigen detection by ELISA +++</u>	<u>Serum, whole blood, skin biopsy</u>	<u>DSe 67%–100% and DSp 98.8% to 100% reported</u>			<u>Relatively simple to perform, rapid, can be cost-effective and suitable for high-throughput applications. There is no need for cell culture facility.</u>	<u>Maternal antibodies in colostrum may interfere with testing for antigen in serum using ELISA in calves. Ear notch samples are less affected. PI calves <i>in utero</i> defies detection.</u>	
<u>NA detection by (real-time) RT-PCR +++</u>	<u>Blood; nasal, oral or conjunctival swab (in cases of respiratory disease) or faecal swab (enteric disease)</u>	<u>Depending on the assay analytical sensitivity of less than 10 genome copies/reaction</u>		<u>See reference</u>	<u>- Very sensitive - Rapid - High-throughput - Well established internationally - Depending on the assay, detects all BVDV species - Allows assay-dependent for differentiation of BVDV types 1 and 2 - Detects persistent and transient infection - Proficiency panel of different Pestivirus strains available - Detection of viral RNA in skin biopsy samples unaffected from maternally derived antibodies</u>	<u>- Possibility for contamination at sample collection or in laboratory, leading to false positive results - Needs specialised equipment</u>	<u>- Hoffmann <i>et al.</i> (2006). <i>J. Virol. Methods</i>, 136, 200–209.</u>
<u>Antigen detection by IHC ++</u>	<u>Fixed tissues or frozen sections for Ag detection or NA if using ISH</u>	<u>Lower DSe than other methods; high DSp</u>	<u>N/A</u>	<u>N/A</u>	<u>Allows visualisation of viral components in lesions and assessment of tissue distribution</u>	<u>Technically demanding, limited reagents for detection of Ag in formalin-fixed tissues</u>	
<u>Antibody detection by ELISA +</u>	<u>Paired serum samples, fetal fluids (blood,</u>	<u>DSe and DSp may differ depending on the ELISA used (commercial/in-</u>			<u>- Simple to perform and cost-effective.</u>	<u>- Some cross-reactivity with antibodies induced by other pestiviruses.</u>	

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population used to measure accuracy</u>	<u>Validation report</u>	<u>Advantages: expert opinion</u>	<u>Disadvantages: expert opinion</u>	<u>References</u>
	pericardial, thoracic	house) and the antibodies being tested (e.g. antibodies against structural (E2) and non-structural (NS2-3) proteins.			<ul style="list-style-type: none"> - Can be used to differentiate between acute and persistent infections by demonstration of seroconversion in acute infections - Detection of antibodies in aborted fetuses, stillborn animals can confirm <i>in utero</i> infection in second half of gestation 	- PI animals are usually seronegative (in both of the paired samples)	

12 N/A: not available

Appendix 5: Bovine viral diarrhoea
Intended purpose of test: prevalence of infection – surveillance

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population used to measure accuracy</u>	<u>Validation report</u>	<u>Advantages: expert opinion</u>	<u>Disadvantages: expert opinion</u>	<u>References</u>
Antigen detection by ELISA +++	Serum, whole blood	DSe 67–100% and DSp 98.8–100% reported			Relatively simple to perform, rapid, very cost-effective and suitable for high-throughput applications. There is no need for cell culture facility.	Maternal antibodies in colostrum may interfere with testing for antigen in serum using ELISA in calves. Ear notch samples are less affected. PI calves <i>in utero</i> defies detection.	Sarrazin <i>et al.</i> (2013). <i>Prev. Vet. Med.</i> , 108 , 28–37
NA detection by (real-time) RT-PCR +++	Ear notch (skin), blood, milk		Whole Swiss, German and Irish cattle populations	See references	<ul style="list-style-type: none"> - Very sensitive - Rapid - High-throughput - Well established internationally - Depending on the assay, detects all BVDV species - Allows assay-dependent for differentiation of BVDV types 1 and 2 - Detects persistent and transient infection - Proficiency panel of different Pestivirus strains available - Detection of viral RNA in skin biopsy samples unaffected from maternally-derived antibodies 	<ul style="list-style-type: none"> - Possibility for contamination at sample collection or in laboratory, leading to false positive results - Needs specialised equipment 	<ul style="list-style-type: none"> - Presi & Heim (2010). <i>Vet. Microbiol.</i>, 142, 137–142 - Schweizer <i>et al.</i> (2021). <i>Front. Vet. Sci.</i>, 8, 702730 - Wernike <i>et al.</i> (2017) <i>Pathogens</i>, 6 (4) - Graham <i>et al.</i> (2021). <i>Front. Vet. Sci.</i>, 8, 674557
Antibody detection by ELISA +++	Bulk milk, blood	DSe and DSp may differ depending on the ELISA used (commercial/in-house) and the antibodies being tested (e.g. antibodies against structural (E2) and non-structural (NS2-3) proteins.			<ul style="list-style-type: none"> - Simple to perform and cost-effective - Milk collection is non-invasive method 	<ul style="list-style-type: none"> - Some cross-reactivity with antibodies induced by vaccines and other pestiviruses. - PI animal will be seronegative - Bulk milk from herd excludes males, non-lactating or young stock. 	Barrett <i>et al.</i> (2022). <i>BMC Vet Res.</i> , 18 , 210.
Virus neutralisation test +	Serum	DSe & DSp both extremely high, both >99%. Historical reference serological test.	N/A	Historical information with no formal validation	<ul style="list-style-type: none"> - Very high specificity - Allows differentiation of antibodies to BVDV species 	<ul style="list-style-type: none"> - ASe can vary depending on virus strain used - Requires cell culture, good quality samples - Takes 5 days to obtain results. Labour intensive - not amenable to testing very large numbers of samples. 	N/A

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population used to measure accuracy</u>	<u>Validation report</u>	<u>Advantages: expert opinion</u>	<u>Disadvantages: expert opinion</u>	<u>References</u>
						- No differentiation between infected and vaccinated animals	

15 N/A: not available

16
17

Appendix 6: Bovine viral diarrhoea

Intended purpose of test: immune status in individual animals or populations (post-vaccination)

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population used to measure accuracy</u>	<u>Validation report</u>	<u>Advantages: expert opinion</u>	<u>Disadvantages: expert opinion</u>	<u>References</u>
<u>Antibody detection by ELISA +++</u>	<u>Individual milk, bulk milk, blood (antibodies present against structural and non-structural proteins)</u>	<u>DSe and DSp differs based on the ELISA used (commercial/in-house) and the antibodies being tested (e.g. antibodies against structural (E2) and non-structural (NS2-3) proteins.</u>			<ul style="list-style-type: none"> - Simple to perform and cost-effective - Milk collection is non-invasive method 	<ul style="list-style-type: none"> - Some cross-reactivity with antibodies induced by vaccines and other pestiviruses. - While a DIVA capability is preferred, this is very difficult to achieve using the combinations of existing vaccines and serological assays. Live attenuated vaccines preclude a DIVA capability unless genetically modified to include a 'marker' gene or deletion that can be detected by a serological assay. - PI animal will be seronegative - Bulk milk from herd excludes males, non-lactating or young stock 	<u>Raue et al. (2011). Vet. J., 187, 330–334.</u> <u>Gonzalez et al., (2014). Vet. J., 199, 424–428.</u> <u>Sayers et al., (2015). Vet. J., 205, 56–61.</u>
<u>Virus neutralisation test +++</u>	<u>Serum</u>	<u>DSe & DSp both extremely high, both >99%. Historical reference serological test.</u>	<u>N/A</u>	<u>Historical information with no formal validation</u>	<ul style="list-style-type: none"> - Very high specificity - Good correlation with immunity - Can provide a measure of cross protection between BVDV species 	<ul style="list-style-type: none"> - ASe can vary depending on virus strain used - Requires cell culture, good quality samples - Labour intensive, takes 5 days to obtain results - No differentiation between infected and vaccinated animals 	<u>N/A</u>

18 N/A: not available

Annexe 12. Chapter 3.4.12 ‘Lumpy skin disease’

CHAPTER 3.4.12.

LUMPY SKIN DISEASE

SUMMARY

Description of the disease: Lumpy skin disease (LSD) is a poxvirus disease of cattle characterised by fever, nodules on the skin, mucous membranes and internal organs, emaciation, enlarged lymph nodes, oedema of the skin, and sometimes death. The disease is of economic importance as it can cause a temporary reduction in milk production, temporary or permanent sterility in bulls, damage to hides and, occasionally, death. Various strains of capripoxvirus are responsible for the disease. These are antigenically indistinguishable from strains causing sheep pox and goat pox yet distinct at the genetic level. LSD has a partially different geographical distribution from sheep and goat pox, suggesting that cattle strains of capripoxvirus do not infect and transmit between sheep and goats. Transmission of LSD virus (LSDV) is thought to be predominantly by arthropods, natural contact transmission in the absence of vectors being inefficient. Lumpy skin disease is endemic in ~~most~~ many African and Middle Eastern countries. Between 2012 and 2022, LSD spread into south-east Europe, the Balkans, Russia and Asia as part of the Eurasian LSD epidemic.

Pathology: the nodules are firm and may extend to the underlying subcutis and muscle. Acute histological key lesions consist of epidermal vacuolar changes with intracytoplasmic inclusion bodies and dermal vasculitis. Chronic key histological lesions consist of fibrosis and necrotic sequestrae.

Detection of the agent: Laboratory confirmation of LSD is most rapid using a real-time or conventional polymerase chain reaction (PCR) method specific for capripoxviruses in combination with a clinical history of a generalised nodular skin disease and enlarged superficial lymph nodes in cattle. Ultrastructurally, capripoxvirus virions are distinct from those of parapoxvirus, which causes bovine papular stomatitis and pseudocowpox, but cannot be distinguished morphologically from orthopoxvirus virions, including cowpox and vaccinia viruses, both of which can cause disease in cattle, although neither causes generalised infection and both are uncommon in cattle. LSDV will grow in tissue culture of bovine, ovine or caprine origin. In cell culture, LSDV causes a characteristic cytopathic effect and intracytoplasmic inclusion bodies that is distinct from infection with Bovine herpesvirus 2, which causes pseudo-lumpy skin disease and produces syncytia and intranuclear inclusion bodies in cell culture. Capripoxvirus antigens can be demonstrated in tissue culture using immunoperoxidase or immunofluorescent staining and the virus can be neutralised using specific antisera.

A variety of conventional and real-time PCR tests as well as isothermal amplification tests using capripoxvirus-specific primers have been published for use on a variety of samples.

Serological tests: The virus neutralisation test (VNT) and enzyme-linked immunosorbent assays (ELISAs) are widely used and have been validated. The agar gel immunodiffusion test and indirect immunofluorescent antibody test are less specific than the VNT due to cross-reactions with antibody to other poxviruses. Western blotting using the reaction between the P32 antigen of LSDV with test sera is both sensitive and specific, but is difficult and expensive to carry out.

41 **Requirements for vaccines:** All strains of capripoxvirus examined so far, whether derived from
42 cattle, sheep or goats, are antigenically similar. Attenuated cattle strains, and strains derived from
43 sheep and goats have been used as live vaccines against LSDV.

44 A. INTRODUCTION

45 Lumpy skin disease (LSD) was first seen in Zambia in 1929, spreading into Botswana by 1943 (Haig, 1957),
46 and then into South Africa the same year, where it affected over eight million cattle causing major economic
47 loss. In 1957 it entered Kenya, ~~at the same time as~~ associated with an outbreak of sheep pox (Weiss, 1968).
48 In 1970 LSD spread north into the Sudan, by 1974 it had spread west as far as Nigeria, and in 1977 was
49 reported from Mauritania, Mali, Ghana and Liberia. Another epizootic of LSD between 1981 and 1986 affected
50 Tanzania, Kenya, Zimbabwe, Somalia and the Cameroon, with reported mortality rates in affected cattle of
51 20%. The occurrence of LSD north of the Sahara desert and outside the African continent was confirmed for
52 the first time in Egypt and Israel between 1988 and 1989, and was reported again in 2006 (Brenner *et al.*,
53 2006). In the past decade, LSD occurrences have been reported in the Middle Eastern, European and Asian
54 regions (for up-to-date information, consult WOAAH WAHIS interface¹). Lumpy skin disease outbreaks tend to
55 be sporadic, depending upon animal movements, immune status, and wind and rainfall patterns affecting
56 vector populations. The principal method of transmission is thought to be mechanical by various arthropod
57 vectors (Tuppurainen *et al.*, 2015).

58 Lumpy skin disease virus (LSDV) belongs to the family *Poxviridae*, subfamily *Chordopoxvirinae*
59 *Chordopoxviridae*, and genus *Capripoxvirus*. In common with other poxviruses LSDV replicates in the
60 cytoplasm of an infected cell, forming distinct perinuclear viral factories. The LSD virion is large and brick-
61 shaped measuring 293–299nm (length) and 262–273nm (width). The LSDV genome structure is also similar
62 to other poxviruses, consisting of double-stranded linear DNA that is 25% GC-rich, approximately 150,000 bp
63 in length, and encodes around 156 open reading frames (ORFs). An inverted terminal repeat sequence of
64 2200–2300 bp is found at each end of the linear genome. The linear ends of the genome are joined with a
65 hairpin loop. The central region of the LSDV genome contains ORFs predicted to encode proteins required for
66 virus replication and morphogenesis and exhibit a high degree of similarity with genomes of other mammalian
67 poxviruses. The ORFs in the outer regions of the LSDV genome have lower similarity and likely encode
68 proteins involved in viral virulence and host range determinants.

69 Phylogenetic analysis shows the majority of LSDV strains group into two monophyletic clusters (cluster 1.1
70 and 1.2) (Biswas *et al.*, 2020; Van Schalkwyk *et al.*, 2021). Cluster 1.1 consists of LSDV Neethling vaccine
71 strains that are based on the LSDV/Neethling/LW-1959 vaccine strain (Kara *et al.*, 2003; Van Rooyen *et al.*,
72 1959; van Schalkwyk *et al.*, 2020) and historic wild-type strains from South Africa. Cluster 1.2 consists of wild-
73 type strains from southern Africa, Kenya, the northern hemisphere, and the Kenyan KSGP O-240 commercial
74 vaccine. In addition to these two clusters, there have recently been recombinant LSDV strains isolated from
75 clinical cases of LSD in the field in Russia and central Asia (Flannery *et al.*, 2021; Sprygin *et al.*, 2018; 2020;
76 Wang *et al.*, 2021). These recombinant viruses show unique patterns of accessory gene alleles, consisting of
77 sections of both wild-type and “vaccine” LSDV strains.

78 The severity of the clinical signs of LSD is highly variable and depends on a number of factors, including the
79 strain of capripoxvirus, the age of the host, immunological status and breed. *Bos taurus* is generally more
80 susceptible to clinical disease than *Bos indicus*; the Asian buffalo (*Bubalus spp.*) has also been reported to be
81 susceptible. Within *Bos taurus*, the fine-skinned Channel Island breeds develop more severe disease, with
82 lactating cows appearing to be the most at risk. However, even among groups of cattle of the same breed kept
83 together under the same conditions, there is a large variation in the clinical signs presented, ranging from
84 subclinical infection to death (Carn & Kitching, 1995). There may be failure of the virus to infect the whole group,
85 probably depending on the virulence of the virus isolate, immunological status of the host, host genotype, and
86 vector prevalence. Seroprevalence studies, experimental infections and case reports have provided indications
87 that several wildlife species (e.g. springbok, impala, giraffe, camel, banteng) are susceptible to LSDV infection
88 (Dao *et al.*, 2022; Hedger & Hamblin, 1983; Kumar *et al.*, 2023; Porco *et al.*, 2023). The scarcity of documented
89 outbreaks in wildlife and the fact that available studies remain limited in number and mostly involve only a few
90 animals, make it difficult to determine the role of wildlife in LSDV epidemiology. This topic deserves further study.

¹ <https://www.woah.org/en/what-we-do/animal-health-and-welfare/disease-data-collection/>

91 especially given the current spread of LSDV in new geographical areas where large numbers of naïve, potentially
 92 susceptible wild bovines and other ruminants are present.

93 The incubation period under field conditions has not been reported, but following experimental inoculation is
 94 6–9 days until the onset of fever. In the acutely infected animal, there is an initial pyrexia, which may exceed
 95 41°C and persist for 1 week. All the superficial lymph nodes become enlarged. In lactating cattle there is a
 96 marked reduction in milk yield. Lesions develop over the body, particularly on the head, neck, udder, scrotum,
 97 vulva and perineum between 7 and 19 days after virus inoculation (Coetzer, 2004). The characteristic
 98 integumentary lesions are multiple, well circumscribed to coalescing, 0.5–5 cm in diameter, firm, flat-topped
 99 papules and nodules. The nodules involve the dermis and epidermis, and may extend to the underlying
 100 subcutis and occasionally to the adjacent striated muscle. These nodules have a creamy grey to white colour
 101 on cut section, which may initially exude serum, but over the ensuing 2 weeks a cone-shaped central core or
 102 sequestrum of necrotic material/necrotic plug (“sit-fast”) may appear within the nodule. The acute histological
 103 lesions consist of epidermal vacuolar changes with intracytoplasmic inclusion bodies and dermal vasculitis.
 104 The inclusion bodies are numerous, intracytoplasmic, eosinophilic, homogenous to occasionally granular and
 105 they may occur in endothelial cells, fibroblasts, macrophages, pericytes, and keratinocytes. The dermal lesions
 106 include vasculitis with fibrinoid necrosis, oedema, thrombosis, lymphangitis, dermal-epidermal separation, and
 107 mixed inflammatory infiltrate. The chronic lesions are characterised by an infarcted tissue with a sequestered
 108 necrotic core, often rimmed by granulation tissue gradually replaced by mature fibrosis. At the appearance of
 109 the nodules, the discharge from the eyes and nose becomes mucopurulent, and keratitis may develop.
 110 Nodules may also develop in the mucous membranes of the mouth and alimentary tract, particularly the
 111 abomasum and in the trachea and the lungs, resulting in primary and secondary pneumonia. The nodules on
 112 the mucous membranes of the eyes, nose, mouth, rectum, udder and genitalia quickly ulcerate, and by then
 113 all secretions, ocular and nasal discharge and saliva contain LSD virus (LSDV). The limbs may be oedematous
 114 and the animal is reluctant to move. Pregnant cattle may abort, and there is a report of intrauterine transmission
 115 (Rouby & Aboulsoudb, 2016). Bulls may become permanently or temporarily infertile and the virus can be
 116 excreted in the semen for prolonged periods (Irons *et al.*, 2005). Recovery from severe infection is slow; the
 117 animal is emaciated, may have pneumonia and mastitis, and the necrotic plugs of skin, which may have been
 118 subject to fly strike, are shed leaving deep holes in the hide (Prozesky & Barnard, 1982).

119 The main differential diagnosis is pseudo-LSD caused by bovine herpesvirus 2 (BoHV-2). This is usually a
 120 milder clinical condition, characterised by superficial nodules, resembling only the early stage of LSD. Intra-
 121 nuclear inclusion bodies and viral syncytia are histopathological characteristics of BoHV-2 infection not seen
 122 in LSD. Other differential diagnoses (for integumentary lesions) include: dermatophilosis, dermatophytosis,
 123 bovine farcy, photosensitisation, actinomycosis, actinobacillosis, urticaria, insect bites, besnoitiosis,
 124 nocardiosis, demodicosis, onchocerciasis, pseudo-cowpox, and cowpox. Differential diagnoses for mucosal
 125 lesions include: foot and mouth disease, bluetongue, mucosal disease, malignant catarrhal fever, infectious
 126 bovine rhinotracheitis, and bovine papular stomatitis.

127 LSDV is not transmissible to humans. However, all laboratory manipulations must be performed at an
 128 appropriate containment level determined using biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity:*
 129 *Standard for managing biological risk in the veterinary laboratory and animal facilities*).

130 B. DIAGNOSTIC TECHNIQUES

131 **Table 1.** Test methods available for the diagnosis of LSD 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
Detection of the agent						
Virus isolation	+	++	+	+++	+	–

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
PCR	++	+++	++	+++	+	–
TEM	–	–	–	+	–	–
Detection of immune response						
VNT	++	++	++	++	++	++
IFAT	+	+	+	+	+	+
ELISA	++	++	++	++	++	++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.

PCR = polymerase chain reaction; TEM = Transmission electron microscopy; VNT = virus neutralisation test;
IFAT = indirect fluorescent antibody test; ELISA = enzyme-linked immunosorbent assay.

132
133
134
135

136 1. Detection of the agent

137 1.1. Specimen collection, submission and preparation

138 Material for virus isolation and antigen detection should be collected as biopsies or from skin nodules
139 at post-mortem examination. Samples for virus isolation should preferably be collected within the first
140 week of the occurrence of clinical signs, before the development of neutralising antibodies (Davies,
141 1991; Davies *et al.*, 1971), however virus can be isolated from skin nodules for at least 3–4 weeks
142 thereafter. Samples for genome detection using conventional or real-time polymerase chain reaction
143 (PCR) may be collected when neutralising antibody is present. Following the first appearance of the
144 skin lesions, the virus can be isolated for up to 35 days and viral nucleic acid can be demonstrated
145 via PCR for up to 3 months (Tuppurainen *et al.*, 2005; Weiss, 1968). Buffy coat from blood collected
146 into heparin or EDTA (ethylene diamine tetra-acetic acid) during the viraemic stage of LSD (before
147 generalisation of lesions or within 4 days of generalisation), can also be used for virus isolation.
148 Samples for histology should include the lesion and tissue from the surrounding (non-lesion) area,
149 be a maximum size of 2 cm³, and be placed immediately following collection into ten times the sample
150 volume of 10% neutral buffered formal saline.

151 Tissues in formalin have no special transportation requirements in regard to biorisks. Blood samples
152 with anticoagulant for virus isolation from the buffy coat should be placed immediately on ice after
153 gentle mixing and processed as soon as possible. In practice, the samples may be kept at 4°C for
154 up to 2 days prior to processing, but should not be frozen or kept at ambient temperatures. Tissues
155 for virus isolation and antigen detection should be kept at 4°C, on ice or at –20°C. If it is necessary
156 to transport samples over long distances without refrigeration, the medium should contain 10%
157 glycerol; the samples should be of sufficient size (e.g. 1 g in 10 ml) that the transport medium does
158 not penetrate the central part of the biopsy, which should be used for virus isolation.

159 ~~Samples for histology should include the lesion and tissue from the surrounding (non-lesion) area,~~
160 ~~be a maximum size of 2 cm³, and be placed immediately following collection into ten times the sample~~
161 ~~volume of 10% neutral buffered formaldehyde. Tissues in formalin have no special transportation~~
162 ~~requirements in regard to biorisks.~~ Material for histology should be prepared using standard
163 techniques and stained with haematoxylin and eosin (H&E) (Burdin, 1959). Lesion material for virus
164 isolation and antigen detection is minced using a sterile scalpel blade and forceps and then
165 macerated in a sterile steel ball-bearing mixer mill, or ground with a pestle in a sterile mortar with
166 sterile sand and an equal volume of sterile phosphate buffered saline (PBS) or serum-free modified
167 Eagle's medium containing sodium penicillin (1000 international units [IU]/ml), streptomycin sulphate

168 (1 mg/ml), mycostatin (100 IU/ml) or fungizone (amphotericin, 2.5 µg/ml) and neomycin (200 IU/ml).
169 The suspension is freeze–thawed three times and then partially clarified using a bench centrifuge at
170 600 **g** for 10 minutes. In cases where bacterial contamination of the sample is expected (such as
171 when virus is isolated from skin samples), the supernatant can be filtered through a 0.45 µm pore
172 size filter after the centrifugation step. Buffy coats may be prepared from unclotted blood using
173 centrifugation at 600 **g** for 15 minutes, and the buffy coat carefully removed into 5 ml of cold double-
174 distilled water using a sterile Pasteur pipette. After 30 seconds, 5 ml of cold double-strength growth
175 medium is added and mixed. The mixture is centrifuged at 600 **g** for 15 minutes, the supernatant is
176 discarded and the cell pellet is suspended in 5 ml growth medium, such as Glasgow’s modified
177 Eagle’s medium (GMEM). After centrifugation at 600 **g** for a further 15 minutes, the resulting pellet
178 is suspended in 5 ml of fresh GMEM. Alternatively, the buffy coat may be separated from a
179 heparinised sample by using a Ficoll gradient.

180 **1.2. Virus isolation on cell culture**

181 LSDV will grow in tissue culture of bovine, ovine or caprine origin. MDBK (Madin–Darby bovine
182 kidney) cells are often used, as they support good growth of the virus and are well characterised
183 (Fay *et al.*, 2020). Primary cells, such as lamb testis (LT) cells also support viral growth, but care
184 needs to be taken to ensure they are not contaminated with viruses such as bovine viral diarrhoea
185 virus. One ml of clarified supernatant or buffy coat is inoculated onto a confluent monolayer in a
186 25 cm² culture flask at 37°C and allowed to adsorb for 1 hour. The culture is then washed with warm
187 PBS and covered with 10 ml of a suitable medium, such as GMEM, containing antibiotics and 2%
188 fetal calf serum. If available, tissue culture tubes containing appropriate cells and a flying cover-slip,
189 or tissue culture microscope slides, are also infected.

190 The flasks/tissue culture tubes are examined daily for 7–14 days for evidence of cytopathic effects
191 (CPE). Infected cells develop a characteristic CPE consisting of retraction of the cell membrane from
192 surrounding cells, and eventually rounding of cells and margination of the nuclear chromatin. At first
193 only small areas of CPE can be seen, sometimes as soon as 2 days after infection; over the following
194 4–6 days these expand to involve the whole cell monolayer-sheet. If no CPE is apparent by day 14,
195 the culture should be freeze–thawed three times, and clarified supernatant inoculated on to a fresh
196 cell monolayer. At the first sign of CPE in the flasks, or earlier if a number of infected cover-slips are
197 being used, a cover-slip should be removed, fixed in acetone and stained using H&E. Eosinophilic
198 intracytoplasmic inclusion bodies, which are variable in size but up to half the size of the nucleus and
199 surrounded by a clear halo, are diagnostic for poxvirus infection. PCR may be used as an alternative
200 to H&E for confirmation of the diagnosis. The CPE can be prevented or delayed by adding specific
201 anti-LSDV serum to the medium. In contrast, the herpesvirus that causes pseudo-LSD produces a
202 Cowdry type A intranuclear inclusion body. It also forms syncytia.

203 An ovine testis cell line (OA3.Ts) has been evaluated for the propagation of capripoxvirus isolates
204 (Babiuk *et al.*, 2007), however this cell line has been found to be contaminated with pestivirus and
205 should be used with caution.

206 **1.3. Polymerase chain reaction (PCR)**

207 The conventional gel-based PCR method described below is a simple, fast and sensitive method for
208 the detection of capripoxvirus genome in EDTA blood, semen or tissue culture samples (Tuppurainen
209 *et al.*, 2005).

210 **1.3.1. Test procedure**

211 The extraction method described below can be replaced using commercially available DNA
212 extraction kits.

- 213 i) Freeze and thaw 200 µl of blood in EDTA, semen or tissue culture supernatant and
214 suspend in 100 µl of lysis buffer containing 5 M guanidine thiocyanate, 50 mM potassium
215 chloride, 10 mM Tris/HCl (pH 8); and 0.5 ml Tween 20.

- 216 ii) Cut skin and other tissue samples into fine pieces using a sterile scalpel blade and
 217 forceps. Grind with a pestle in a mortar. Suspend the tissue samples in 800 µl of the
 218 above mentioned lysis buffer.
- 219 iii) Add 2 µl of proteinase K (20 mg/ml) to blood samples and 10 µl of proteinase K
 220 (20 mg/ml) to tissue samples. Incubate at 56°C for 2 hours or overnight, followed by
 221 heating at 100°C for 10 minutes. Add phenol:chloroform:isoamylalcohol (25:24:1 [v/v]) to
 222 the samples in a 1:1 ratio. Vortex and incubate at room temperature for 10 minutes.
 223 Centrifuge the samples at 16,060 **g** for 15 minutes at 4°C. Carefully collect the upper,
 224 aqueous phase (up to 200 µl) and transfer into a clean 2.0 ml tube. Add two volumes of
 225 ice cold ethanol (100%) and 1/10 volume of 3 M sodium acetate (pH 5.3). Place the
 226 samples at –20°C for 1 hour. Centrifuge again at 16,060 **g** for 15 minutes at 4°C and
 227 discard the supernatant. Wash the pellets with ice cold 70% ethanol (100 µl) and
 228 centrifuge at 16,060 **g** for 1 minute at 4°C. Discard the supernatant and dry the pellets
 229 thoroughly. Suspend the pellets in 30 µl of nuclease-free water and store immediately at
 230 –20°C (Tuppurainen *et al.*, 2005). Alternatively a column-based extraction kit may be
 231 used.
- 232 iv) The primers for this PCR assay were developed from the gene encoding the viral
 233 attachment protein. The size of the expected amplicon is 192 bp (Ireland & Binopal,
 234 1998). The primers have the following gene sequences:
- 235 Forward primer 5'-TCC-GAG-CTC-TTT-CCT-GAT-TTT-TCT-TAC-TAT-3'
- 236 Reverse primer 5'-TAT-GGT-ACC-TAA-ATT-ATA-TAC-GTA-AAT-AAC-3'.
- 237 v) DNA amplification is carried out in a final volume of 50 µl containing: 5 µl of 10 × PCR
 238 buffer, 1.5 µl of MgCl₂ (50 mM), 1 µl of dNTP (10 mM), 1 µl of forward primer, 1 µl of
 239 reverse primer, 1 µl of DNA template (~10 ng), 0.5 µl of *Taq* DNA polymerase and 39 µl
 240 of nuclease-free water. The volume of DNA template required may vary and the volume
 241 of nuclease-free water must be adjusted to the final volume of 50 µl.
- 242 vi) Run the samples in a thermal cycler as follows: 2 minutes at 95°C; then 45 seconds at
 243 95°C, 50 seconds at 50°C and 1 minute at 72°C (34 cycles); 2 minutes at 72°C and hold
 244 at 4°C until analysis.
- 245 vii) Mix 10 µl of each sample with loading dye and load onto a 1.5% agarose gel in TAE buffer
 246 (Tris/acetate buffer containing EDTA). Load a parallel lane with a 100 bp DNA-marker
 247 ladder. Electrophoretically separate the products using approximately 8–10 V/cm for 40–
 248 60 minutes and visualise with a suitable DNA stain and transilluminator.

249 Quantitative real-time PCR methods have been described that are reported to be faster and have
 250 higher sensitivity than conventional PCRs (Balinsky *et al.*, 2008; Bowden *et al.*, 2008). A real-time
 251 PCR method that differentiates between LSDV, sheep pox virus and goat pox virus has been
 252 published (Lamien *et al.*, 2011).

253 Quantitative real-time PCR assays have been designed to differentiate between Neethling-based
 254 LSDV strains, which are often used for vaccination, and wild-type LSDV strains from cluster 1.2
 255 (Agianniotaki *et al.*, 2017; Pestova *et al.*, 2018; Vidanovic *et al.*, 2016). These “DIVA” assays (DIVA:
 256 differentiation of infected from vaccinated animals) enable, for example, differentiation of “Neethling
 257 response” caused by vaccination with a LSDV Neethling vaccine strain from disease caused by
 258 infection with a cluster 1.2 wild-type virus. However these DIVA PCR assays cannot distinguish
 259 between a LSDV Neethling vaccine strain and the novel recombinant LSDV strains ~~recently~~ isolated
 260 from disease outbreaks in Asia (Byadovskaya *et al.*, 2024; Flannery *et al.*, 2021). These DIVA assays
 261 are also not capable of discriminating between LSDV Neethling vaccine strains and recently
 262 characterised (historic) wild-type viruses from South Africa belonging within cluster 1.1 (Van
 263 Schalkwyk *et al.*, 2020; 2021). Consequently, in regions where recombinant strains (currently Asia
 264 and possibly elsewhere) or wild-type cluster 1.1 strains are circulating (currently South Africa
 265 and possibly elsewhere), these DIVA assays are not suitable for distinguishing vaccine and wild-type
 266 virus. Thus, in order to overcome these constraints, whole genome sequencing is recommended.

267 **1.4. Transmission electron microscopy**

268 The characteristic poxvirus virion can be visualised using a negative staining preparation technique
269 followed by examination with an electron microscope. There are many different negative staining
270 protocols, an example of which is given below.

271 **1.4.1. Test procedure**

272 Before centrifugation, material from the original biopsy suspension is prepared for examination
273 under the transmission electron microscope by floating a 400-mesh hexagonal electron
274 microscope grid, with pioloform-carbon substrate activated by glow discharge in pentylamine
275 vapour, onto a drop of the suspension placed on parafilm or a wax plate. After 1 minute, the
276 grid is transferred to a drop of Tris/EDTA buffer, pH 7.8, for 20 seconds and then to a drop of
277 1% phosphotungstic acid, pH 7.2, for 10 seconds. The grid is drained using filter paper, air-
278 dried and placed in the electron microscope. The capripox virion is brick shaped, covered in
279 short tubular elements and measures approximately 290 × 270 nm. A host-cell-derived
280 membrane may surround some of the virions, and as many as possible should be examined
281 to confirm their appearance (Kitching & Smale, 1986).

282 The ~~capripox~~ virions of capripoxvirus are indistinguishable from those of orthopoxvirus, but, apart
283 from vaccinia virus and cowpox virus, which are both uncommon in cattle and do not cause
284 generalised infection, no other orthopoxvirus is known to cause lesions in cattle. However, vaccinia
285 virus may cause generalised infection in young immunocompromised calves. In contrast,
286 orthopoxviruses are a common cause of skin disease in domestic buffalo (*Bubalus bubalis*) causing
287 buffalo pox, a disease that usually manifests as pock lesions on the teats, but may cause skin lesions
288 at other sites, such as the perineum, the medial aspects of the thighs and the head. Orthopoxviruses
289 that cause buffalo pox cannot be readily distinguished from capripoxvirus by electron microscopy.
290 The virions of parapoxvirus ~~virions~~ that cause bovine papular stomatitis and pseudocowpox are
291 smaller, oval in shape and each is covered in a single continuous tubular element that appears as
292 striations over the virion. Capripoxvirus virions are also distinct from the herpesvirus that causes
293 pseudo-LSD (also known as "Allerton" or bovine herpes mammillitis).

294 **1.5. Fluorescent antibody tests**

295 Capripoxvirus antigen can be identified on infected cover-slips or tissue culture slides using
296 fluorescent antibody tests. Cover-slips or slides should be washed and air-dried and fixed in cold
297 acetone for 10 minutes. The indirect test using immune cattle sera is subject to high background
298 colour and nonspecific reactions. However, a direct conjugate can be prepared from sera from
299 convalescent cattle (or from sheep or goats convalescing from capripox) or from rabbits
300 hyperimmunised with purified capripoxvirus. Uninfected tissue culture should be included as a
301 negative control as cross-reactions can cause problems due to antibodies to cellular components
302 (pre-absorption of these from the immune serum helps solve this issue).

303 **1.6. Immunohistochemistry**

304 Immunohistochemistry using F80G5 monoclonal antibody specific for capripoxvirus ORF 057 has
305 been described for detection of LSDV antigen in the skin of experimentally infected cattle (Babiuk *et*
306 *al.*, 2008).

307 **1.7. Isothermal genome amplification**

308 Molecular tests using loop-mediated isothermal amplification to detect capripoxvirus genomes are
309 reported to provide sensitivity and specificity similar to real-time PCR with a simpler method and
310 lower cost (Das *et al.*, 2012; Murray *et al.*, 2013). Field validation of the Das *et al.* method was
311 reported by Omoga *et al.* (2016).

312 2. Serological tests

313 All the viruses in the genus *Capripoxvirus* share a common major antigen for neutralising antibodies and
314 it is thus not possible to distinguish strains of capripoxvirus from cattle, sheep or goats using serological
315 techniques.

316 2.1. Virus neutralisation

317 A test serum can either be titrated against a constant titre of capripoxvirus (100 TCID₅₀ [50% tissue
318 culture infective dose]) or a standard virus strain can be titrated against a constant dilution of test
319 serum in order to calculate a neutralisation index. Because of the variable sensitivity of tissue culture
320 to capripoxvirus, and the consequent difficulty of ensuring the accurate and repeatable seeding of
321 100 TCID₅₀/well, the neutralisation index is the preferred method in most laboratories, although it
322 does require a larger volume of test sera. The test is described using 96-well flat-bottomed tissue-
323 culture grade microtitre plates, but it can be performed equally well in tissue culture tubes with the
324 appropriate changes to the volumes used, although it is more difficult to read an end-point in tubes.

325 2.1.1. Test procedure

- 326 i) Test sera, including a negative and a positive control, are diluted 1/5 in Eagle's/HEPES
327 (N-2-hydroxyethylpiperazine, N-2-ethanesulphonic acid) buffer and inactivated at 56°C
328 for 30 minutes.
- 329 ii) Next, 50 µl of the first inactivated serum is added to columns 1 and 2, rows A to H of the
330 microtitre plate. The second serum is placed in columns 3 and 4, the third in columns 5
331 and 6, the positive control serum is placed in columns 7 and 8, the negative control serum
332 is placed in columns 9 and 10, and 50 µl of Eagle's/HEPES buffer (without serum) is
333 placed in columns 11 and 12, and to all wells in row H.
- 334 iii) A reference strain of capripoxvirus, usually a vaccine strain known to grow well in tissue
335 culture, with a titre of over log₁₀ 6 TCID₅₀ per ml is diluted in Eagle's/HEPES in bijoux
336 bottles to give a log dilution series of log₁₀ 5.0, 4.0, 3.5, 3.0, 2.5, 2.0, 1.5 TCID₅₀ per ml
337 (equivalent to log₁₀ 3.7, 2.7, 2.2, 1.7, 1.2, 0.7, 0.2 TCID₅₀ per 50 µl).
- 338 iv) Starting with row G and the most diluted virus preparation, 50 µl of virus is added to each
339 well in that row. This is repeated with each virus dilution, the highest titre virus dilution
340 being placed in row A.
- 341 v) The plates are covered and incubated for 1 hour at 37°C.
- 342 vi) An appropriate cell suspension (such as MDBK cells) is prepared from pregrown
343 monolayers as a suspension of 10⁵ cells/ml in Eagle's medium containing antibiotics and
344 2% fetal calf serum. Following incubation of the microtitre plates, 100 µl of cell suspension
345 is added to all the wells, except wells H11 and H12, which serve as control wells for the
346 medium. The remaining wells of row H are cell and serum controls.
- 347 vii) The microtitre plates are covered and incubated at 37°C for 9 days.
- 348 viii) Using an inverted microscope, the monolayers are examined daily from day 4 for
349 evidence of CPE. There should be no CPE in the cells of row H. Using the 0240 KSGP
350 vaccine strain of capripoxvirus, by way of example, the final reading is taken on day 9,
351 and the titre of virus in each duplicate titration is calculated using the Kärber method. If
352 left longer, there is invariably a 'breakthrough' of virus in which virus that was at first
353 neutralised appears to disassociate from the antibody.
- 354 ix) *Interpretation of the results:* The neutralisation index is the log titre difference between
355 the titre of the virus in the negative serum and in the test serum. An index of ≥1.5 is
356 positive. The test can be made more sensitive if serum from the same animal is examined
357 before and after infection. Because the immunity to capripoxviruses is predominantly cell
358 mediated, a negative result, particularly following vaccination, after which the antibody
359 response may be low, does not imply that the animal from which the serum was taken is
360 not protected.

361 Antibodies to capripoxvirus can be detected from 1 to 2 days after the onset of clinical
362 signs. These remain detectable for about 7 months.

363 2.2. Enzyme-linked immunosorbent assay

364 Enzyme-linked immunosorbent assays (ELISAs) for the detection of capripoxviral antibodies are
365 widely used and are available in commercial kit form (Milovanovic *et al.*, 2019; Samojlovic *et al.*,
366 2019).

367 2.3. Indirect fluorescent antibody test

368 Capripoxvirus-infected tissue culture grown on cover-slips or tissue culture microscope slides can be
369 used for the indirect fluorescent antibody test. Uninfected tissue culture control, and positive and
370 negative control sera, should be included in the test. The infected and control cultures are fixed in
371 acetone at -20°C for 10 minutes and stored at 4°C . Dilutions of test sera are made in PBS, starting
372 at 1/20 or 1/40, and positive samples are identified using an anti-bovine gamma-globulin conjugated
373 with fluorescein isothiocyanate. Antibody titres may exceed 1/1000 after infection. Sera may be
374 screened at 1/50 and 1/500. Cross-reactions can occur with orf virus (contagious pustular dermatitis
375 virus of sheep), bovine papular stomatitis virus and perhaps other poxviruses.

376 2.4. Western blot analysis

377 Western blotting of test sera against capripoxvirus-infected cell lysate provides a sensitive and
378 specific system for the detection of antibody to capripoxvirus structural proteins, although the test is
379 expensive and difficult to carry out.

380 Capripoxvirus-infected LT cells should be harvested when 90% CPE is observed, freeze-thawed
381 three times, and the cellular debris pelleted using centrifugation. The supernatant should be
382 decanted, and the proteins should be separated using SDS/PAGE (sodium dodecyl
383 sulphate/polyacrylamide gel electrophoresis). A vertical discontinuous gel system, using a stacking
384 gel made up of acrylamide (5%) in Tris (125 mM), pH 6.8, and SDS (0.1%), and a resolving gel made
385 up of acrylamide (10–12.5%) in Tris (560 mM), pH 8.7, and SDS (0.1%), is recommended for use
386 with a glycine running buffer containing Tris (250 mM), glycine (2 M), and SDS (0.1%). Samples of
387 supernatant should be prepared by boiling for 5 minutes with an appropriate lysis buffer prior to
388 loading. Alternatively, purified virus or recombinant antigens may replace tissue-culture-derived
389 antigen.

390 Molecular weight markers should be run concurrently with the protein samples. The separated
391 proteins in the SDS/PAGE gel should be transferred electrophoretically to a nitrocellulose membrane
392 (NCM). After transfer, the NCM is rinsed thoroughly in PBS and blocked in 3% bovine serum albumin
393 (BSA) in PBS, or 5% skimmed milk powder in PBS, on a rotating shaker at 4°C overnight. The NCM
394 can then be separated into strips by employing a commercial apparatus to allow the concurrent
395 testing of multiple serum samples, or may be cut into strips and each strip incubated separately
396 thereafter. The NCM is washed thoroughly with five changes of PBS for 5 minutes on a rotating
397 shaker, and then incubated at room temperature on the shaker for 1.5 hours, with the appropriate
398 serum at a dilution of 1/50 in blocking buffer (3% BSA and 0.05% Tween 20 in PBS; or 5% milk
399 powder and 0.05% Tween 20 in PBS). The membrane is again thoroughly washed and incubated (in
400 blocking buffer) with anti-species immunoglobulin horseradish-peroxidase-conjugated
401 immunoglobulins at a dilution determined using titration. After further incubation at room temperature
402 for 1.5 hours, the membrane is washed and a solution of diaminobenzidine tetrahydrochloride (10 mg
403 in 50 ml of 50 mM Tris/HCl, pH 7.5, and 20 μl of 30% [v/v] hydrogen peroxide) is added.
404 Incubation is then undertaken for approximately 3–7 minutes at room temperature on a shaker with
405 constant observation, and the reaction is stopped by washing the NCM in PBS before excessive
406 background colour is seen. A positive and negative control serum should be used on each occasion.

407 Positive test samples and the positive control will produce a pattern consistent with reaction to
408 proteins of molecular weights 67, 32, 26, 19 and 17 kDa – the major structural proteins of
409 capripoxvirus – whereas negative serum samples will not react with all these proteins. Hyperimmune

410 serum prepared against parapoxvirus (bovine papular stomatitis or pseudocowpox virus) will react
411 with some of the capripoxvirus proteins, but not the 32 kDa protein that is specific for capripoxvirus.

412 C. REQUIREMENTS FOR VACCINES

413 1. Background: rationale and intended use of the product

414 ~~Live attenuated strains of capripoxvirus have been used as vaccines specifically for the control of LSD (Brenner~~
415 ~~et al., 2006; Capstick & Coakley, 1961; Carn, 1993). Capripoxviruses are cross-reactive within the genus.~~
416 ~~Consequently, it is possible to protect cattle against LSD using strains of capripoxvirus derived from sheep or~~
417 ~~goats (Coakley & Capstick, 1961). However, it is recommended to carry out controlled trials, using the most~~
418 ~~susceptible breeds, prior to introducing a vaccine strain not usually used in cattle. The duration of protection~~
419 ~~provided by LSD vaccination is unknown.~~

420 ~~Capripoxvirus vaccine strains can produce a large local reaction at the site of inoculation in *Bos taurus* breeds~~
421 ~~(Davies, 1991), which some stock owners find unacceptable. This has discouraged the use of vaccine, even~~
422 ~~though the consequences of an outbreak of LSD are invariably more severe. Risk-benefit of vaccination should~~
423 ~~be assessed following stakeholder discussion.~~

424 Vaccines are a key tool to control LSD. Different types of LSD vaccines have been developed and several are
425 commercially available (Tuppurainen et al., 2021).

426 Live attenuated vaccines (LAV) based on the Neethling LSDV strain (homologous LAV vaccines) have been
427 shown to offer high levels of protection against LSD under experimental conditions (Haegeman et al., 2021)
428 and have been used successfully to control the disease in the field, through systematic vaccination of the entire
429 country's cattle population for a number of consecutive years (Klement et al., 2020). Homologous vaccines
430 may induce fever, produce a local reaction at the site of inoculation, cause a temporary reduction in milk
431 production and on rare occasions induce a 'Neethling' response (Ben-Gera et al., 2015; Davies, 1991;
432 Haegeman et al., 2021). Such adverse effects, however, usually resolve within a few days and are largely
433 outweighed by the overall benefits of vaccination with homologous vaccines. The duration of immunity induced
434 by good quality live attenuated LSDV vaccines was shown to be at least 18 months (Haegeman et al., 2023).

435 As capripoxviruses provide cross-reactive protection within the genus, heterologous LAVs comprising
436 sheeppox virus or goatpox virus strains have also been tested and used to protect cattle against LSD.
437 Sheeppox virus-based heterologous vaccines usually contain higher doses of virus than when administered
438 to sheep. Although safe, their effectiveness in protecting cattle against LSD is inferior compared to homologous
439 vaccines (Ben-Gera et al., 2015; Zhugunissoff et al., 2020). Heterologous vaccines containing goatpox virus
440 strains for use in cattle against LSD have been developed more recently. One such vaccine based on the
441 Gorgan strain provided protection under experimental conditions comparable to homologous vaccines (Gari et
442 al., 2015). On the other hand, a goat pox vaccine based on an attenuated Uttarkashi goatpox virus strain
443 performed suboptimally under field conditions in India (Naveem et al., 2023), indicating that further research
444 is warranted before asserting that all goatpox virus-based vaccines induce protection equal to homologous
445 vaccines in cattle against LSD.

446 In addition, homologous inactivated vaccines against LSD have been developed and tested (Haegeman et al.,
447 2023; Hamdi et al., 2020; Wolff et al., 2022). These vaccines are reported to be safe and efficacious. They
448 however require a booster vaccination one month after primo-vaccination and then every 6 months thereafter,
449 based on the fact that the duration of immunity is shorter than 1 year (Haegeman et al., 2023).

450 None of the commercial vaccines currently available has practical DIVA capacity. This problem may be
451 resolved in the future by introducing new types of vaccines (e.g. vector-vaccines, subunit vaccines, mRNA
452 vaccines) that are at various stages of development and evaluation.

453 **2. Outline of production of LSD vaccines and minimum requirements for conventional**
454 **vaccines**

455 ~~General requirements set for the facilities used for the production of vaccines and for the documentation and~~
456 ~~record keeping throughout the whole manufacturing process are described in Chapter 1.1.8 *Principles of*~~
457 ~~*veterinary vaccine production*. The documentation should include standard operating procedures (SOP) for~~
458 ~~the method of manufacture and each step for the testing of cells and reagents used in the process, each batch~~
459 ~~and the final product.~~

460 The production of vaccines, including LSD vaccines, starts within research and development (R&D) facilities
461 where vaccine candidates are produced and tested in preclinical studies to demonstrate the quality, safety and
462 efficacy of the product.

463 Minimum requirements for different production stages of veterinary vaccines are available in different chapters
464 of the *Terrestrial Manual*. These are intended to be used in combination with country-specific regulatory
465 requirements for vaccine production and release. Here we outline the most important requirements for the
466 production of live and inactivated LSD vaccines. Full requirements are available in Chapter 1.1.8 *Principles of*
467 *veterinary vaccine production*, Chapter 2.3.3 *Minimum requirements for the organisation and management of*
468 *a vaccine manufacturing facility* and Chapter 2.3.4 *Minimum requirements for the production and quality control*
469 *of vaccine*, and other regulatory documentation.

470 **2.1. Quality assurance**

471 Facilities for manufacturing LSD vaccines should operate in line with the concepts of good laboratory
472 practice (GLP) and good manufacturing practice (GMP) to produce high quality products. Quality risk
473 management and quality control with adequate documentation management, as an integral part of
474 the production process, have to be in place. In case some activities of the production process are
475 outsourced, those should also be appropriately defined, recorded and controlled.

476 The vaccine production process (Outline of Production) should be documented in a series of
477 standard operating procedures (SOPs), or other documents describing the manufacturing of each
478 batch and the final product (including starting materials to be used, manufacturing steps, in-process
479 controls and controls on the final product). Detailed requirements for documentation management in
480 the process of vaccine production are available in Chapter 2.3.3.

481 A completed Outline of Production is to be enclosed in a vaccine candidate dossier and used for the
482 evaluation of the production process and product by regulatory bodies.

483 **2.2. Process validation**

484 The dossier with the enclosed Outline of Production for the vaccine candidate has to be submitted
485 for regulatory approval, so it can be assessed and authorised by the competent authority to ensure
486 compliance with local regulatory requirements. Among others, data on quality, safety, and efficacy
487 will be assessed. The procedures necessary to obtain these data are described in the subsequent
488 sections.

489 National regulatory authorities might also require official control authority re-testing (check testing)
490 of final products and batches in government laboratories or an independent batch quality control by
491 a third party.

492 **3. Requirements for LSD vaccine candidates and batch production**

493 **3.1. Requirements for starting materials**

494 Live attenuated vaccines (LAV) and inactivated vaccines (IV) for LSD are produced using the system
495 of limited and controlled passages of master seed and working seed virus and cell banks with a
496 specified maximum. This approach aims to prevent possible and unwanted drift of properties of seed
497 virus and cells that might arise from repeated passaging.

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3.1.1. Characteristics of the seed virus

~~Each seed strain of capripoxvirus used for vaccine production must be accompanied by records clearly and accurately describing its origin, isolation and tissue culture or animal passage history. Preferably, the species and strain of capripoxvirus are characterised using PCR or DNA sequencing techniques.~~

~~A quantity of master seed vaccine virus should be prepared, frozen or desiccated and stored at low temperatures such as -80°C and used to produce a consistent working seed for regular vaccine production.~~

~~Each master seed strain must be non-transmissible, remain attenuated after further tissue culture passage, and provide complete protection against challenge with virulent field strains for a minimum of 1 year. It must produce a minimal clinical reaction in cattle when given via the recommended route.~~

~~The necessary safety and potency tests are described in Section C.2.2.4 *Final product batch tests*.~~

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

~~Each master seed must be tested to ensure its identity and shown to be free from adventitious viruses, in particular pestiviruses, such as border disease and bovine viral diarrhoea virus, and free from contamination with bacteria, fungi or mycoplasmas.~~

~~The general procedures for sterility or purity tests are described in Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*.~~

Master seed virus is a quantity of virus of uniform composition derived from an original isolate, passaged for a documented number of times and distributed into containers at one time and stored adequately to ensure stability (via freezing or lyophilisation). Selection of master seed viruses (MSVs) should ideally be based on their ease of growth in cell culture, virus yield, and in accordance with the regional epidemiological importance. Also, measures to minimise transmissible spongiform encephalopathies (TSE) contamination should be taken into account (see Section C.3.5.1 *Purity tests*).

For each seed strain selected for LSD vaccine production, the following information should be provided:

- Historical record: geographical origin, animal species from which the virus was recovered, isolation procedure, tissue culture or animal passage history
- Identity: species and strain identification using DNA sequencing
- Purity: the absence of bacteria, fungi, mycoplasma, and other viruses (see Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*)
- Safety (overdose, one/repeated dose tests, and reversion to virulence tests) (see Section C.3.3 *Vaccine safety*)
- Efficacy data, linked to a specified (protective) dose (see Section C.3.4 *Vaccine efficacy*)
- Stability

Each master seed strain selected for production of live attenuated LSD vaccines must remain attenuated after further passage in animals (see Section C.3.3. *Vaccine safety*), produce minimal clinical reaction when given via the recommended route, provide complete protection against challenge with virulent field strains, and is ideally not transmissible.

A quantity of master seed virus should be prepared and stored to be further used for the preparation of working seeds and production seeds. Working seed viruses may be expanded

543 in one or more (but, limited) cell culture passages from the master seed stock and used to
544 produce vaccine batches. This approach and limitation of seed virus passaging will assist in
545 maintaining uniformity and consistency in production.

546 **3.1.2. Master cell stocks**

547 The production process of LSD vaccines ideally employs an established master cell stock
548 (MCS) system with defined lowest and highest cell passage to be used to grow the vaccine
549 virus. Primary cells derived from normal tissues can be used in the production process, but
550 the use of primary cells has an inherently higher risk of introducing extraneous agents
551 compared with the use of established (well characterised) cell lines and should be avoided
552 where alternative methods of producing effective vaccines exist. For each MCS,
553 manufacturers should demonstrate:

- 554 - MCS identity
- 555 - genetic stability by subculturing from the lowest to the highest passage used for
556 production
- 557 - stable MCS karyotype with a low level of polyploidy
- 558 - freedom from oncogenicity or tumorigenicity by using *in-vivo* studies using the highest
559 cell passage that may be used for production
- 560 - purity of MCSs from extraneous bacteria, fungi, mycoplasma, and viruses
- 561 - implemented measures to lower TSE contamination risk (see Section C.3.5.1 *Purity*
562 *tests*).

563 **3.2. Method of vaccine manufacturing**

564 The method of manufacture should be documented as the Outline of Production.

565 **2.2.1. Procedure**

566 **3.2.1. LSD vaccine batch production**

567 ~~Vaccine batches are produced on an appropriate cell line such as MDBK. As already~~
568 ~~mentioned in the first paragraphs of Section C, all steps undertaken in the production of~~
569 ~~vaccine batches should be described and documented in the Outline of Production. The~~
570 ~~production of LAV and IV against LSD starts with the inoculation of the required number of~~
571 ~~working vials of seed virus is reconstituted with GMEM or other in appropriate medium and~~
572 ~~inoculated onto a suitable primary or continuous cell line grown in suspension or monolayer.~~
573 ~~Cells should be harvested after 4–8 days when they exhibit 50–70% CPE for maximum in the~~
574 ~~exponential growth phase. At the time highest viral infectivity, or earlier if CPE is extensive~~
575 ~~and cells appear ready to detach. Techniques such as loads are present, sonication or~~
576 ~~repeated freeze–thawing are is used to release the intracellular virus from the cytoplasm. The~~
577 ~~lysate may then be clarified using centrifugation to remove cellular debris (for example by use~~
578 ~~of centrifugation at 600 g for 20 minutes, with retention of the supernatant). A second passage~~
579 ~~of the virus may be required to produce sufficient virus for a production batch.~~

580 An aliquot of the virus suspension is titrated to check the virus titre. For LAV, the virus-
581 containing suspension is diluted to attain the dose at which the vaccine candidate will be
582 evaluated or to at least the determined protective dose for approved vaccines and is then
583 mixed with a suitable protectant such as an equal volume of sterile, chilled 5% lactalbumin
584 hydrolysate and 10% sucrose (dissolved in double distilled water or appropriate balanced salt
585 solution), and transferred to individually numbered-labelled bottles or bags for storage at low
586 temperatures such as –80°C, or for freeze–drying. A written record of all the procedures
587 followed must be kept for all vaccine batches.

588 **2.2.2. Requirements for substrates and media**

589 The specification and source of all ingredients used in the manufacturing procedure should be
590 documented and the freedom of extraneous agents (bacteria, fungi, mycoplasma and viruses)
591 should be tested. The detailed testing procedure is described in Chapter 1.1.9. The use of
592 antibiotics must meet the requirements of the licensing authority.

593 **2.2.3. In-process control**

594 i) Cells

595 Records of the source of the master cell stocks should be maintained. The highest and
596 lowest passage numbers of the cells that can be used for vaccine production must be
597 indicated in the Outline of the Production. The use of a continuous cell line (such as
598 MDBK, etc.) is strongly recommended, unless the virus strain only grows on primary cells.
599 The key advantage of continuous over primary cell lines is that there is less risk of
600 introduction of extraneous agents.

601 ii) Serum

602 Serum used in the growth or maintenance medium must be free from antibodies to
603 capripoxvirus and free from contamination with pestivirus or other viruses, extraneous
604 bacteria, mycoplasma or fungi.

605 iii) Medium

606 Media must be sterile before use.

607 iv) Virus

608 Seed virus and final vaccine must be titrated and pass the minimum release titre set by
609 the manufacturer. For example, the minimum recommended field dose of the South
610 African Neethling strain vaccines (Mathijs *et al.*, 2016) is \log_{10} 3.5 TCID₅₀, although the
611 minimum protective dose is \log_{10} 2.0 TCID₅₀. Capripoxvirus is highly susceptible to
612 inactivation by sunlight and allowance should be made for loss of activity in the field.

613 The recommended field dose of the Romanian sheep pox vaccine for cattle is \log_{10}
614 2.5 sheep infective doses (SID₅₀), and the recommended dose for cattle of the RM65-
615 adapted strain of Romanian sheep pox vaccine is \log_{10} 3 TCID₅₀ (Coakley & Capstick,
616 1961).

617 **3.2.2. Inactivation process for inactivated LSD vaccines**

618 Unlike LAV, inactivated LSD vaccines contain inactivated antigens in combination with
619 adjuvants to strengthen the induced immune response after administration. The vaccine
620 evaluation process described below needs to show the amount of antigen necessary to elicit
621 a protective immune response. Currently, literature data indicate that an inactivated vaccine
622 originating from an LSDV virus stock with titre 10^4 cell culture infectious dose₅₀ (CCID₅₀)/ml
623 before inactivation can be sufficient to induce an efficient immune response to prevent clinical
624 disease, viremia and virus shedding after challenge of young cattle (Wolf *et al.*, 2022)

625 To monitor the inactivation process and the level of antigen inactivation, samples are taken at
626 regular intervals during inactivation and titrated. Inactivation conditions and the length of initial
627 and repeated exposure should be documented in detail since one or more factors during the
628 process could influence the outcomes. The inactivation kinetics should reach a predefined
629 target e.g. one remaining infectious unit per million doses (1×10^{-6} infectious units/dose) as
630 suggested by APHIS (2013). The confirmatory testing of inactivation is performed on each
631 vaccine lot and represents an important part of the inactivation process monitoring. In addition
632 to all the procedures mentioned above, the inactivation procedure and tests demonstrating
633 that antigen inactivation is complete and consistent must additionally be documented in the
634 Outline of Production.

635

3.3. Vaccine safety

636 During the vaccine development process, vaccine safety must be evaluated in the target animal
637 (target animal batch safety test –TABST) to demonstrate the safety of the dose intended for
638 registration. The animals used in the safety testing should be representative (species, age and
639 category [calves, heifers, bulls, cows.]) for all the animals for which the vaccine is intended.
640 Vaccinated and control groups are appropriately acclimatised, housed and managed in line with
641 animal welfare standards. Animal suffering has to be eliminated or reduced and euthanasia is
642 recommended in moribund animals.

643 Essential parameters to be evaluated in safety studies are local and systemic reactions to
644 vaccination, including local reactions at the site of administration, fever, effect on milk production,
645 and induction of a 'Neethling' response. The effect of the vaccine on reproduction needs to be
646 evaluated where applicable.

647 A part of the safety evaluation of LAV and IV can be performed during the efficacy trials (see Section
648 C.3.4 Vaccine efficacy) by measuring local and systemic responses following vaccination and before
649 challenge.

650 Guidelines for safety evaluation are provided by the European Medicine Agency (EMA) in VICH
651 GL44: TABST for LAV and IV (EMA, 2009). Safety aspects of LAV and IV against LSD to be
652 evaluated are:

3.3.1. Overdose test for LAV

654 Local and systemic responses should be measured following an overdose test whereby 10×
655 the maximum vaccine titre is administered. If the maximum vaccine titre is not specified, 10×
656 the minimum vaccine titre can be applied in multiple injection sites. Ideally, the 10× dose is
657 dissolved in the 1× dose volume of the adjuvants or diluent. Generally, eight animals per group
658 should be used (EMA, 2009).

3.3.2. One dose and repeat dose test

660 This aims to test the safety of the vaccine dose applied in the vaccination regime intended for
661 registration. LAV LSD vaccines require one dose per year, while inactivated LSD vaccines
662 require a booster dose in addition to the primary dose. The minimal recommended interval
663 between administrations is 14 days.

664 Generally, eight animals per group should be used unless otherwise justified (EMA, 2009).
665 For each target species, the most sensitive breed, age and sex proposed on the label should
666 be used. Seronegative animals should be used. In cases where seronegative animals are not
667 reasonably available, alternatives should be justified.

3.3.3. Reversion to virulence tests

669 Live attenuated vaccines inherently carry the risk of vaccine virus reverting to virulence when
670 repeated passages in a host species could occur due to shedding and transmission from
671 vaccinated animals to contact animals. LAV LSD vaccines should therefore be tested for non-
672 reversion to virulence by means of passage studies. Vaccine virus (MSV, not the finished
673 vaccine) is inoculated in a group of target animals of susceptible age via the natural route of
674 infection or the route that is most likely to result in infection. The vaccine virus is subsequently
675 recovered from tissues or excretions and is used directly to inoculate a further group of
676 animals. After not less than four passages (see chapter 1.1.8), i.e. use of a total of five groups
677 of animals, the re-isolate must be fully characterised, using the same procedures used to
678 characterise the master seed virus.

3.3.4. Environmental consideration

680 This includes the evaluation of the ability of LAV LSD vaccines to be shed, to spread and to
681 infect contact target and non-target animals, and to persist in the environment.

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2.2.4. Final product batch tests

i) ~~Sterility/purity~~

~~Vaccine samples must be tested for sterility/purity. Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in Chapter 1.1.9.~~

ii) ~~Safety and efficacy~~

~~The efficacy and safety studies should be demonstrated using statistically valid vaccination challenge studies using seronegative young LSDV susceptible dairy cattle breeds. The group numbers recommended here can be varied if statistically justified. Fifteen cattle are placed in a high containment level large animal unit and serum samples are collected. Five randomly chosen vials of the freeze-dried vaccine are reconstituted in sterile PBS and pooled. Two cattle are inoculated with 10 times the recommended field dose of the vaccine, and eight cattle are inoculated with the recommended field dose. The remaining five cattle are unvaccinated control animals. The animals are clinically examined daily and rectal temperatures are recorded. On day 21 after vaccination, the animals are again serum sampled and challenged with a known virulent capripoxvirus strain. The challenge virus solution should also be tested free from extraneous viruses. The clinical response is recorded during the following 14 days. Animals in the unvaccinated control group should develop the typical clinical signs of LSD, whereas there should be no local or systemic reaction in the vaccinates other than a raised area in the skin at the site of vaccination, which should disappear after 4 days. Serum samples are again collected on day 30 after vaccination. The day 21 serum samples are examined for seroconversion to selected viral diseases that could have contaminated the vaccine, and the days 0 and 30 samples are compared to confirm the absence of antibody to pestivirus. Because of the variable response in cattle to LSD challenge, generalised disease may not be seen in all of the unvaccinated control animals, although there should be a large local reaction.~~

~~Once the efficacy of the particular strain being used for vaccine production has been determined in terms of minimum dose required to provide immunity, it is not necessary to repeat this on the final product of each batch, provided the titre of virus present has been ascertained.~~

iii) ~~Batch potency~~

~~Potency tests in cattle must be undertaken for vaccine strains of capripoxvirus if the minimum immunising dose is not known. This is usually carried out by comparing the titre of a virulent challenge virus on the flanks of vaccinated and control animals. Following vaccination, the flanks of at least three animals and three controls are shaved of hair. Log₁₀ dilutions of the challenge virus are prepared in sterile PBS and six dilutions are inoculated intradermally (0.1 ml per inoculum) along the length of the flank; four replicates of each dilution are inoculated down the flank. An oedematous swelling will develop at possibly all 24 inoculation sites on the control animals, although preferably there will be little or no reaction at the four sites of the most dilute inocula. The vaccinated animals may develop an initial hypersensitivity reaction at sites of inoculation within 24 hours, which should quickly subside. Small areas of necrosis may develop at the inoculation site of the most concentrated challenge virus. The titre of the challenge virus is calculated for the vaccinated and control animals; a difference in titre >log₁₀ 2.5 is taken as evidence of protection.~~

3.4. Vaccine efficacy

Data enclosed in the vaccine candidate dossier should support the efficacy of the vaccine in each animal species for 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 and for the duration of immunity. Efficacy studies should be conducted with the vaccine candidate that has been

733 produced at the highest passage level permitted for vaccine production as specified in the Outline of
734 Production.

735 Efficacy (and safety) should be demonstrated in vaccination–challenge studies using representative
736 (by species, age and category) seronegative healthy animals for which the vaccine is intended and
737 which are tested negative for standard viral pathogens.

738 An example of a vaccination-challenge test set-up is outlined here. The group numbers mentioned
739 can be varied if statistically justified. Thirteen animals are placed in a high containment large animal
740 unit and are divided into two groups:

741 - single/repeated dose test group (n=8) – animals inoculated with the vaccine dose and route
742 intended for registration (in case of an IV against LSD, a booster dose should follow primary
743 vaccination after minimum 14 days).

744 - control group (n=5) – non-vaccinated animals

745 Throughout the *in-vivo* study, all animals are clinically examined and rectal temperatures recorded.
746 Blood, serum and swab samples are regularly collected and subjected to laboratory testing. On day
747 21 after the vaccination with a LAV or after the booster vaccination for an IV, the animals in both
748 groups are challenged with a known virulent LSDV strain. The challenge virus solution should be of
749 known titre and tested free from extraneous viruses. Experience obtained from previous animal
750 experiments indicates that a dose of challenge virus between 10^{4.0} and 10^{6.5} TCID₅₀ produces clinical
751 disease in about half of the susceptible experimental cattle (Tuppurainen *et al.*, 2021).

752 The clinical response following challenge is recorded over a period of 14 days. No clinical signs
753 should occur in the vaccinates, other than a local reaction at the site of inoculation. At least 1 animal
754 in the unvaccinated control group should develop the typical clinical signs of LSD. Although a
755 generalised disease with skin nodules may not be seen in all the unvaccinated control animals based
756 on the knowledge that the outcome of a LSDV infection can range from inapparent to severe, at the
757 very least a large local reaction is to be expected.

758 Clinical and laboratory results will enable assessment of the safety and efficacy of the LSD vaccine
759 candidate and the induced immune responses. Serum samples collected at different time points
760 during the trial can be examined to study seroconversion against selected viral diseases that could
761 have contaminated the vaccine.

762 **2.3. Requirements for regulatory approval**

763 **2.3.1. Safety requirements**

764 i) ~~Target and non-target animal safety~~

765 ~~The vaccine must be safe to use in all breeds of cattle for which it is intended, including~~
766 ~~young and pregnant animals. It must also be non-transmissible and remain attenuated~~
767 ~~after further tissue culture passage.~~

768 ~~Safety tests should be carried out on the final product of each batch as described in~~
769 ~~Section C.2.2.4.~~

770 ii) ~~Reversion to virulence for attenuated/live vaccines~~

771 ~~The selected final vaccine should not revert to virulence during further passages in target~~
772 ~~animals.~~

773 iii) ~~Environmental consideration~~

774 ~~Attenuated vaccine should not be able to perpetuate autonomously in a cattle population.~~
775 ~~Strains of LSDV are not a hazard to human health.~~

776 **2.3.2. Efficacy requirements**

777 i) — For animal production

778 The efficacy of the vaccine must be demonstrated in statistically valid vaccination
779 challenge experiments under laboratory conditions. The group numbers recommended
780 here can be varied if statistically justified. Fifteen cattle are placed in a high containment
781 level large animal unit and serum samples are collected. Five randomly chosen vials of
782 the freeze-dried vaccine are reconstituted in sterile PBS and pooled. Two cattle are
783 inoculated with 10 times the field dose of the vaccine, eight cattle are inoculated with the
784 recommended field dose. The remaining five cattle are unvaccinated control animals. The
785 animals are clinically examined daily and rectal temperatures are recorded. On day 21
786 after vaccination, the animals are again serum sampled and challenged with a known
787 virulent capripoxvirus strain using intravenous and intradermal inoculation (the challenge
788 virus solution should also be tested and shown to be free from extraneous viruses). The
789 clinical response is recorded during the following 14 days. Animals in the unvaccinated
790 control group should develop the typical clinical signs of LSD, whereas there should be
791 no local or systemic reaction in the vaccinates other than a raised area in the skin at the
792 site of vaccination which should disappear after 4 days. Serum samples are again
793 collected on day 30 after vaccination. The day 21 serum samples are examined for
794 seroconversion to selected viral diseases that could have contaminated the vaccine, and
795 the days 0 and 30 samples are compared to confirm the absence of antibody to pestivirus.
796 Because of the variable response in cattle to challenge with LSDV, generalised disease
797 may not be seen in all of the unvaccinated control animals, although there should be a
798 large local reaction.

799 Once the potency of the particular strain being used for vaccine production has been
800 determined in terms of minimum dose required to provide immunity, it is not necessary to
801 repeat this on the final product of each batch, provided the titre of virus present has been
802 ascertained.

803 ii) — For control and eradication

804 Vaccination is the only effective way to control LSD outbreaks in endemic countries and
805 recent experiences of the disease in Eastern Europe and the Balkans suggests this is
806 also true for outbreaks in non-endemic countries. Unfortunately, currently no marker
807 vaccines allowing a DIVA strategy are available, although to a limited extent PCR can be
808 used for certain vaccines.

809 The duration of immunity produced by LSDV vaccine strains is currently unknown.

810 **2.3.3. Stability**

811 All vaccines are initially given a shelf life of 24 months before expiry. Real-time stability studies
812 are then conducted to confirm the appropriateness of the expiry date. Multiple batches of the
813 vaccine should be re-titrated periodically throughout the shelf-life period to determine the
814 vaccine stability.

815 Properly freeze-dried preparations of LSDV vaccine, particularly those that include a
816 protectant, such as sucrose and lactalbumin hydrolysate, are stable for over 25 years when
817 stored _____ at
818 -20°C and for 2–4 years when stored at 4°C . There is evidence that they are stable at higher
819 temperatures, but no long-term controlled experiments have been reported. No preservatives
820 other than a protectant, such as sucrose and lactalbumin hydrolysate, are required for the
821 freeze-dried preparation.

822 **3.5. Batch/serial tests before release for distribution**

823 Quality tests on MSV and safety and efficacy tests on vaccine candidates are performed during the
824 evaluation process for new LSD vaccines. Once vaccines are approved to be used in the field, it
825 remains important to verify the quality of each vaccine batch produced. An independent batch quality
826 control assessment may be warranted or requested by national or international regulatory authorities.

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3.5.1. Purity test

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Purity is defined by the absence of different contaminants (bacteria, fungi, mycoplasma, and other viruses; see full details in chapter 1.1.9) in the vaccine and its associated diluent/adjuvants. Virus isolation and bacterial culture tests can be used to show freedom from live competent replicating microorganisms, but molecular methods are more rapid and sensitive, but positives can be caused by genome fragments and incompetent replicating microorganisms.

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Besides the contaminants mentioned above, manufacturers should demonstrate implemented measures to minimise the risk of TSE contamination in ingredients of animal origin such as:

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- all ingredients of animal origin in production facilities are from countries recognised as having the lowest possible risk of bovine spongiform encephalopathy

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- tissues or other substances used are themselves recognised as being of low or nil risk of containing TSE agents

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3.5.2. Identity tests

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In addition to identity tests performed on the MSV, the identity tests on final batches aim to demonstrate the presence of only the selected capripoxvirus species and strain in the vaccine as indicated in the Outline of Production and the absence of other strains or members of the genus and any other viral contaminant that might arise during the production process. Identity testing could be assured by using appropriate tests (e.g. PCRs, sanger sequencing, NGS).

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3.5.3. Potency tests

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Standard requirements for potency tests can be found in CFR Title 9 part 113, in the European Pharmacopoeia, and in this Terrestrial Manual.

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3.5.3.1. Live vaccines

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The potency of LAV against LSD can be measured by means of virus titration. The virus titre must, as a rule, be sufficiently greater than that shown to be protective in the efficacy test for the vaccine candidate. This will ensure that at any time prior to the expiry date, the titre will be at least equal to the evaluated protective titre. The titres of currently available commercial homologous LSD vaccines range between 10³ and 10⁴ infectious units/dose (Tuppurainen *et al.*, 2021).

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3.5.3.2. Inactivated LSD vaccines

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For inactivated LSD vaccines, potency tests are performed using vaccination–challenge efficacy studies in animal hosts (see Section C.3.4. *Vaccine efficacy*).

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3.5.4. Safety/efficacy

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Safety and efficacy testing is undertaken during the evaluation process of the vaccine candidate, and also needs to be performed on a number of vaccine batches until robust data are generated in line with international and national regulations. Afterwards, when using a seed lot system in combination with strict implementation of GMP standards and depending on local regulations, TABST could be waived as described in VICH50 and VICH55, providing the titer has been ascertained using potency testing. Batches or serials are considered satisfactory if local and systemic reactions to vaccination are in line with those described in the dossier of the vaccine candidate and product literature.

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3.5.4.1. Field safety/efficacy tests

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Field testing of two or more batches should be performed on all animal categories for which the product is indicated before release of the product for general use (see chapter 1.1.8). The aim of these studies is to demonstrate the safety and efficacy of the product under normal field conditions of animal care and use in different geographical locations where different factors may influence product performance. A protocol for safety/efficacy testing in the field has to be developed with defined observation and recording procedures. However, it is generally more difficult to obtain statistically significant data to demonstrate efficacy under field conditions. Even when properly designed, field efficacy studies may be inconclusive due to uncontrollable outside influences.

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3.5.4.2. Duration of Immunity

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The duration of immunity (DOI) following vaccination should be demonstrated via challenge or the use of a validated serology test. Efficacy testing at the end of the claimed period of protection should be conducted in each species for which the vaccine is indicated or the manufacturer should indicate that the DOI for that species is not known. Likewise, the manufacturer should demonstrate the effectiveness of the recommended booster regime in line with these guidelines, usually by measuring the magnitude and kinetics of the serological response observed.

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3. Vaccines based on biotechnology

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A new generation of capripox vaccines is being developed that uses the LSDV as a vector for the expression and delivery of immune-protective proteins of other ruminant pathogens with the potential for providing dual protection (Boshra *et al.*, 2013; Wallace & Viljoen, 2005), as well as targeting putative immunomodulatory genes for inducing improved immune responses (Kara *et al.*, 2018).

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4. Post-market studies

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4.1. Stability

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Stability testing shall be carried out as specified in Annex II of Regulation (EU) 2019/6 and in the Ph. Eur. 0062: Vaccines for veterinary use, on not fewer than three representative batches providing this mimics the full-scale production described in the application. At the end of shelf-life, sterility has to be re-evaluated using sterility testing or by showing container closure integrity. Multiple batches of the vaccine should be re-titrated periodically throughout the shelf-life period to determine the vaccine stability.

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4.2. Post-marketing surveillance

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After release of a vaccine, its performance under field conditions should continue to be monitored by competent authorities and by the manufacturer itself. Not all listed adverse effects may show up in the clinical trials performed to assess safety and efficacy of the vaccine candidate due to the limited number of animals used. Post-marketing surveillance studies can also provide information on vaccine efficacy when used in normal practice and husbandry conditions, on duration of induced immunity, on ecotoxicity, etc.

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First, a reliable reporting system should be in place to collect consumer complaints and notifications of adverse reactions. Secondly, post-marketing surveillance should be established to investigate whether the reported observations are related to the use of the product and to identify, at the earliest stage, any serious problem that may be encountered from its use and that may affect its future uptake. Vaccinovigilance should be an on-going and integral part of all regulatory programmes for LSD vaccines, especially for live vaccines.

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 1072 108695. doi: 10.1016/j.vetmic.2020.108695.
- 1073 *
- 1074 * *
- 1075 **NB:** There are WOA Reference Laboratories for lumpy skin disease (please consult the WOA Web site:
 1076 <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>).
- 1077 Please contact WOA Reference Laboratories for any further information on
 1078 diagnostic tests, reagents and vaccines for lumpy skin disease
- 1079 **NB:** FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2021.

Annexe 13.Chapter 3.6.9. 'Equine rhinopneumonitis (infection with Varicellovirus equidalpha1)'

CHAPTER 3.6.9.

EQUINE RHINOPNEUMONITIS (INFECTION WITH VARICELLOVIRUS EQUIDALPHA1-EQUID-HERPESVIRUS-1 AND -4)

SUMMARY

Equine rhinopneumonitis (ER) is a collective term for any one of several contagious, clinical disease entities of equids that may occur as a result of infection by either of two closely related herpesviruses, formally known as equid alpha herpesvirus-1 and -4 (EHV-1 and EHV-4). Infection with EHV-1 is listed by WOAHP and is therefore the focus of this chapter. The classification of the virus has been reviewed and EHV-1 is now known as Varicellovirus equidalpha1. For the purposes of the chapter, the acronym EHV-1 will continue to be used. EHV-1 is and EHV-4 are endemic in most domestic equine populations worldwide.

Primary infection by ~~either EHV-1 or EHV-4~~ is characterised by upper respiratory tract disease of varying severity that is related to the age and immunological status of the infected animal. Following viraemia EHV-1 may also cause the more serious complications of abortion, perinatal foal death, or paralytic neurological disease (equine herpesvirus myeloencephalopathy). ~~EHV-4 has been associated with sporadic cases of abortion, but rarely multiple abortions and not the large outbreaks associated with EHV-1.~~ Like other herpesviruses, EHV-1 and 4 induces long-lasting latent infections and can be reactivated following stress or pregnancy. Furthermore, most horses are likely to be re-infected multiple times during their lifetime, often ~~mildly or subclinically~~. ~~Detection of viral DNA or anti-EHV antibodies should therefore be interpreted with care.~~

Identification of the agent: The standard method of identification of EHV-1 and EHV-4 from appropriate clinical or necropsy material is by polymerase chain reaction (PCR), ~~followed by laboratory isolation of the virus in cell culture.~~

Positive identification of viral isolates as EHV-1 or EHV-4 can be achieved by type-specific PCR or sequencing. Viruses can be isolated in equine cell culture from the following sample types: nasal or nasopharyngeal swab extracts taken from horses ~~during the febrile stage of with acute~~ respiratory tract infection, ~~from the placenta, from and~~ liver, lung, spleen, adrenal glands or thymus of aborted fetuses and early foal deaths, and from the leukocyte fraction of the blood of animals ~~with acute during the febrile stage of EHV-1 infection~~. Unlike EHV-4, EHV-1 will also grow in various non-equine cell types such as the RK-13 cell line and this property can be used to distinguish between the two viruses.

A rapid presumptive diagnosis of abortion induced by EHV-1 or (infrequently) EHV-4 can be achieved by direct immunofluorescent detection of viral antigen in cryostat sections of placenta and tissues from aborted fetuses, using a conjugated polyclonal antiserum.

Post-mortem demonstration of histopathological lesions of EHV-1 in placenta and tissues from aborted fetuses, cases of perinatal foal death, or in the central nervous system of neurologically affected animals complements other diagnostic techniques ~~the laboratory diagnosis.~~

41 **Serological tests:** As most horses possess some level of antibody to EHV-1/4, the
42 demonstration of specific antibody in the serum collected from a single blood sample is
43 therefore not confirmation of a positive diagnosis of recent infection. Paired, (acute and
44 convalescent) sera from animals suspected of being infected with EHV-1 or EHV-4 should
45 be tested for a four-fold or greater rise in virus-specific antibody titre by either virus
46 neutralisation (VN) or complement fixation (CF) tests. Neither of these assays is type-
47 specific but both have proven useful for diagnostic purposes especially since the CF
48 antibody response to recent infection is relatively short-lived. Limited use has also been
49 made of a type-specific enzyme-linked immunosorbent assay (Crabb et al., 1995; Hartley
50 et al., 2005).

51 **Requirements for vaccines:** Both live attenuated and inactivated viral vaccines are
52 available for use in assisting in the control of EHV-1/4. Vaccination is helpful in reducing
53 the severity of respiratory infection in young horses and the incidence of abortion in mares;
54 however current vaccines are not licenced to protect against neurological disease.
55 Vaccination should not be considered a substitute for sound management practices known
56 to reduce the risk of infection. Revaccination at frequent intervals is recommended in the
57 case of each of the products, as the duration of vaccine-induced immunity is relatively short.

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

65 A. INTRODUCTION

66 Equine rhinopneumonitis (ER) is a historically derived term that describes a constellation of several
67 disease entities of horses that may include respiratory disease, abortion, neonatal foal pneumonitis, or
68 myeloencephalopathy (Allen & Bryans, 1986; Allen et al., 1999; Bryans & Allen, 1988; Crabb & Studdert,
69 1995). The disease ~~has been is~~ recognised for ~~over 60 years~~ as a threat to the international horse
70 industry, and is caused by either of two members of the *Herpesviridae* family, formerly known as equid
71 alphaherpesvirus-1 and -4 (EHV-1 and EHV-4). The viruses are now classified as Varicellovirus
72 equidalpha1 and Varicellovirus equidalpha4. For the purposes of the chapter, the acronyms EHV-1 and
73 EHV-4 will continue to be used. EHV-1 and EHV-4 are closely related alphaherpesviruses of horses
74 with nucleotide sequence identity within individual homologous genes ranging from 55% to 84%, and
75 amino acid sequence identity from 55% to 96% (Telford et al., 1992; 1998). The two herpesviruses With
76 the exception of EHV-1 in Iceland (Thorsteinsdóttir et al., 2021), the two herpesviruses are considered
77 endemic enzootic in all countries in which large populations of horses are maintained as part of the
78 cultural tradition or agricultural economy. There is no recorded evidence that the two herpesviruses of
79 ER pose any health risks to humans working with the agents. Infection with EHV-1 is listed by WOA
80 and is therefore the focus of this chapter.

81 Viral transmission to cohort animals occurs by inhalation of aerosols of virus-laden respiratory
82 secretions. Morbidity tends to be highest in young horses sharing the same air space. Aborted tissues
83 and placental fluids from infected mares can contain extremely high levels of live virus and represent a
84 major source of infection. Extensive use of vaccines has not eliminated EHV-1 infections, and the world-
85 wide ~~annual~~ financial impact from ~~this these~~ equine pathogens is ~~immense~~ considerable.

86 In horses under 3 years of age, clinical ER usually takes the form of an acute, febrile respiratory illness
87 that spreads rapidly through the group of animals. The viruses infects and multiplies multiply in epithelial
88 cells of the respiratory mucosa. Signs of infection become apparent 2–8 days after exposure to virus,
89 and are characterised by fever, inappetence, depression, and nasal discharge. The severity of
90 respiratory disease varies with the age of the horse and the level of immunity resulting from previous
91 vaccination or natural exposure. Bi-phasic fever, viraemia and complications are more likely with EHV-
92 1 than EHV-4. Subclinical infections with EHV-1/4 are common, even in young animals. Although
93 mortality from uncomplicated ER is rare and complete recovery within 1–2 weeks is the normal outcome,
94 respiratory infection is a frequent and significant cause of interrupted schedules among horses

95 assembled for training, racing, or other equestrian events. Fully protective immunity resulting from
96 infection is of short duration, and convalescent animals are susceptible to reinfection by EHV-1/4 after
97 several months. Although reinfections by the two herpesviruses cause less severe or clinically
98 inapparent respiratory disease, the risks of subsequent abortion or neurological disease remain. Like
99 other herpesviruses, EHV-1/4 causes long-lasting latent infections and latently infected horses
100 represent a potential infection risk for other horses. Virus can be reactivated as a result of stress or
101 pregnancy. The greatest clinical threats to individual breeding, racing, or pleasure horse operations
102 posed by ER are the potential abortigenic and neurological sequelae of EHV-1 respiratory infection. ER
103 abortions occur annually in horse populations worldwide and may be sporadic or multiple. Foals infected
104 in utero may be born alive and die within a few days of birth. EHV-1 neurological disease is less common
105 than abortions but has been recorded all over the world with associated fatalities. Outbreaks result in
106 movement restrictions and, sometimes, cancellation of equestrian events (Couroucé *et al.*, 2023; FEI,
107 2021).

108 Neurological disease, also known as equine herpesvirus myeloencephalopathy, remains an infrequent
109 but serious complication of EHV-1 infection. A single mutation in the DNA polymerase gene (ORF30)
110 has been associated with increased risk of neurological disease, however strains without this change
111 can also cause paralysis (Goodman *et al.*, 2007; Nugent *et al.*, 2006). Strain typing techniques have
112 been employed to identify viruses carrying the neuropathic marker, and it can be helpful to be aware of
113 an increased risk of neurological complications. However, for practical purposes strain typing is not
114 relevant for agent identification, or international trade. Strain typing may be beneficial for implementation
115 of biosecurity measures in the management of outbreaks of equine herpesvirus myeloencephalopathy.

116 Strain typing has been shown to be not reliable for predicting the clinical outcome of EHV-1 infection but
117 can be useful in epidemiological investigations (Garvey *et al.*, 2019; Nugent *et al.*, 2006; Sutton *et al.*,
118 2019).

119 EU: Strain typing has been shown to be unreliable not reliable for predicting the clinical outcome of EHV-
120 1 infection but can be useful in epidemiological investigations (Garvey *et al.*, 2019; Nugent *et al.*, 2006;
121 Sutton *et al.*, 2019)."

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B. DIAGNOSTIC TECHNIQUES

123 Both EHV-1 and EHV-4 is transmitted by the respiratory route and has have the potential to be highly
124 contagious, viruses particularly where large numbers of horses are housed in the same air space. EHV1
125 and the former can cause explosive outbreaks of abortion or neurological disease. Rapid diagnostic
126 methods are therefore essential useful for managing the disease. Real-time polymerase chain reaction
127 (PCR) assays are widely routinely used by diagnostic laboratories worldwide and are both rapid and
128 sensitive. Real-time PCR assays that allow simultaneous testing for EHV-1 and EHV-4 have been
129 developed for both detection of EHV-1 and quantification of viral load have been developed, and have
130 replaced virus isolation has been replaced by real time PCR as the frontline diagnostic test in the
131 majority of laboratories, but Virus isolation can also still be useful, particularly for the detection of
132 viraemia. This is also true of for in cases of EHV-1-associated abortions and neonatal foal deaths, when
133 the high level of virus in the tissues usually produces a cytopathic effect in 1–3 days. Immunohistochemical or immunofluorescent approaches are employed in some laboratories can be
134 extremely useful for rapid diagnosis of EHV-induced abortion from fresh or embedded tissue and are
135 relatively straightforward. Several other techniques based on enzyme-linked immunosorbent assay
136 (ELISA) or nucleic acid hybridisation probes have also been described, however their use is often
137 restricted to specialised laboratories and they are not included here. Virus neutralisation (VN) and
138 complement fixation test (CFT) are the most frequently used serological tests, and seroconversion in
139 paired samples is considered indicative of exposure to virus by natural infection or by vaccination.
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Table 1. Test methods available for the diagnosis of equine rhinopneumonitis-infection with EHV-1 and their purpose

Method	Purpose					
	Population freedom from infection ^(a)	Individual animal freedom from infection prior to movement ^(b)	Contribute to eradication policies ^(c)	Confirmation of clinical cases ^(d)	Prevalence of infection - surveillance ^(e)	Immune status in individual animals or populations post-vaccination ^(f)
Identification of the agent^(g)						
Virus isolation	-	++-±	-	++	-	-
PCR	-	+++	-	+++	-	-
<u>Direct immunofluorescence</u>	≡	≡	≡	±±	≡	≡
Detection of immune response						
VN	±±	±±	≡±	++ ±	+++	+++
ELISA	+	- ±±	≡+	±±	++-±	±±
CFT	-	- ±±	-	++±	-	- ±±±

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Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; - = not appropriate for this purpose.
PCR = polymerase chain reaction; VN = virus neutralisation;
ELISA = enzyme-linked immunosorbent assay; CFT = complement fixation test.

^(a)See Appendix 1 of this chapter for justification table for the scores giving to the tests for this purpose.

^(b)See Appendix 2 of this chapter for justification table for the scores giving to the tests for this purpose.

^(c)No eradication policies exist for equine rhinopneumonitis.

^(d)See Appendix 4 of this chapter for justification table for the scores giving to the tests for this purpose.

^(e)See Appendix 5 of this chapter for justification table for the scores giving to the tests for this purpose.

^(f)See Appendix 6 of this chapter for justification table for the scores giving to the tests for this purpose.

^(g)A combination of agent identification methods applied on the same clinical sample is recommended.

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1. Identification-Detection of the agent

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1.1. Collection and preparation of specimens

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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~~ acute stage of the respiratory disease, and are collected via the nares by sampling the area with a swab of an appropriate size and length for horses. After collection, the swab should be removed and transported immediately to the virology laboratory in 3 ml of cold (not frozen) virus transport medium (e.g. phosphate buffered saline [PBS] or serum-free MEM [minimal essential medium] with antibiotics). Virus infectivity can be prolonged by the addition of bovine serum albumin, fetal calf serum or gelatine to 0.1% (w/v).

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Tissue samples: total DNA can be extracted using a number of commercially available kits and used in PCR to detect viral DNA (described below in Section B.1.2.1). Virus isolation from placenta and fetal tissues from suspect cases of EHV-1 abortion is most successful when performed on aseptically collected samples of placenta, liver, lung, thymus, adrenal glands and spleen. Virus may be isolated from post-mortem cases of EHV-1 neurological disease by culture of samples of brain and spinal cord but such attempts ~~to isolate virus~~ are often unsuccessful; however, they these samples may be useful for PCR testing and pathological examination. Tissue samples should be transported to the laboratory and held at 4°C until

174 inoculated into tissue culture. Samples that cannot be processed within a few hours should be
175 stored at -70°C .

176 Blood: for virus detection by PCR or isolation from blood leukocytes, collect a 10–20 ml sample
177 of blood, using an aseptic technique in citrate, heparin or EDTA [ethylene diamine tetra-acetic
178 acid] anticoagulant. EDTA is the preferred anticoagulant for PCR testing in some laboratories
179 as heparin may inhibit DNA polymerase. The samples should be transported without delay to
180 the laboratory on ice, but not frozen.

181 Cerebrospinal fluid: the detection of EHV-1 DNA in cerebrospinal fluid has been reported in
182 cases of neurological disease.

183 1.2. Virus detection by polymerase chain reaction

184 PCR has become the primary diagnostic method for the detection of EHV-1 and EHV-4 in
185 clinical specimens, paraffin-embedded archival tissue, or inoculated cell cultures (Borchers &
186 Slater, 1993; Lawrence *et al.*, 1994; O'Keefe *et al.*, 1994; Varrasso *et al.*, 2001). A variety of
187 type-specific PCR primers have been designed to distinguish between the presence of EHV-
188 1 and EHV-4. The correlation between PCR and virus isolation techniques for diagnosis of
189 EHV-1 or EHV-4 is high (Varrasso *et al.*, 2001). Diagnosis by PCR is rapid, sensitive, and
190 does not depend on the presence of infectious virus in the clinical sample. For diagnosis of
191 active infection by EHV, PCR methods are routinely used to detect EHV-1 DNA in
192 nasopharyngeal swabs and tissue samples most reliable with tissue samples from aborted
193 fetuses and placental tissue or from nasopharyngeal swabs of foals and yearlings. They are
194 particularly useful in explosive outbreaks of abortion, respiratory or neurological disease, in
195 which a rapid identification and monitoring of the virus spread is critical for guiding
196 management strategies, including movement restrictions. PCR examination of spinal cord and
197 brain tissue, as well as peripheral blood mononuclear cells (PBMC), are important in seeking
198 a diagnosis on a horse with neurological signs (Pronost *et al.*, 2012).

199 Several PCR assays have been published. A nested PCR procedure can be used to
200 distinguish between EHV-1 and EHV-4. A sensitive protocol suitable for clinical or pathological
201 specimens (nasal secretions, blood leukocytes, brain and spinal cord, fetal tissues, etc.) has
202 been described by Borchers & Slater (1993). However, nested PCR methods have a high risk
203 of laboratory cross-contamination, and sensitive rapid one-step PCR tests to detect EHV-1
204 and EHV-4 (e.g. Lawrence *et al.*, 1994) are preferred. The WOA Reference Laboratories use
205 quantitative real-time PCR assays such as those targeting heterologous sequences of major
206 glycoprotein genes to distinguish between EHV-1 and EHV-4. A multiplex real-time PCR
207 targeting glycoprotein B gene of EHV-1 and EHV-4 was described by Diallo *et al.* (2007). PCR
208 protocols have been developed that can differentiate between EHV-1 strains carrying the
209 ORF30 neuropathogenic marker, using both restriction enzyme digestion of PCR products
210 (Fritsche & Borchers, 2011) or by quantitative real-time PCR (Allen *et al.*, 2007, Smith *et al.*,
211 2012). Methods have also been developed to type strains for epidemiological purposes, based
212 on the ORF68 gene (Nugent *et al.*, 2006). The WOA Reference Laboratories employ in-
213 house methods for strain typing, however these protocols have not yet been validated between
214 different laboratories at an international level.

215 Real-time (or quantitative) PCR has become the method of choice for many the majority of
216 diagnostic tests laboratories and provides rapid and sensitive detection of viral DNA. Equine
217 post-mortem tissues from newborn and adult animals or equine fetal tissue from abortions
218 (tissues containing lung, liver, spleen, thymus, adrenal gland and placental tissues) can be
219 used. For respiratory samples, equine nasopharyngeal swabs or deep nasal swabs (submitted
220 in a suitable viral transport medium), buffy coat, tracheal wash (TW) or broncho-alveolar
221 lavage (BAL) are all suitable. DNA should be extracted using an appropriate kit or robotic
222 system.

223 There is no internationally standardised real-time PCR method for EHV-1 but Table 2
224 summarises the primer and probe sequences for some of the most widely used assays. Type-
225 specific PCR primers have been designed to distinguish between the presence of EHV-1 and

226 EHV-4. The optimised thermocycler times and temperatures are documented in the
 227 publications cited.

228 **Table 2. Primer and probe sequences for EHV1/4 detection by real-time PCR**

<u>Primer</u>	<u>Primer sequence (5' to 3')</u>	<u>Target</u>	<u>Reference</u>
<u>Forward</u>	<u>CAT-GTC-AAC-GCA-CTC-CCA</u>	<u>EHV-1 gB</u>	<u>Diallo et al., 2006</u>
<u>Reverse</u>	<u>GGG-TCG-GGC-GTT-TCT-GT</u>		
<u>Probe</u>	<u>FAM-CCC-TAC-GCT-GCT-CC-MGB-NFQ</u>		
<u>Forward</u>	<u>CAT-ACG-TCC-CTG-TCC-GAC-AGA-T</u>	<u>EHV-1 gB</u>	<u>Hussey et al., 2006</u>
<u>Reverse</u>	<u>GGTACTCGGCCTTTGACGAA</u>		
<u>Probe</u>	<u>FAM-TGA-GAC-CGA-AGA-TCT-CCT-CCA-CCG-A-BHQ1</u>		
<u>Forward</u>	<u>TAT-ACT-CGC-TGA-GGA-TGG-AGA-CTT-T</u>	<u>EHV-1 gB</u>	<u>Pusterla et al., 2009</u>
<u>Reverse</u>	<u>TTG-GGG-CAA-GTT-CTA-GGT-GGT-T</u>		
<u>Probe</u>	<u>6FAM-ACA-CCT-GCC-CAC-CGC-CTA-CCG</u>		
<u>Forward</u>	<u>GCG-GGC-TCT-GAC-AAC-ACA-A</u>	<u>EHV-1 gC</u>	ISO 17025 accredited for the detection of EHV-1 at WOAH Reference Laboratory
<u>Reverse</u>	<u>TTG-TGG-TTT-CAT-GGG-AGT-GTG-TA</u>		
<u>Probe</u>	<u>FAM-TAA-CGC-AAA-CGG-TAC-AGA-A-BHQ1</u>		

229 *This multiplex real time PCR test has been validated to ISO 17025 and is designed for use in a 96 well
 230 format. This can be readily combined with automatic nucleic acid extraction methods. Discrimination
 231 between EHV 1 and EHV 4 is carried out by the incorporation of type specific dual labelled probes based
 232 on methods published by Hussey et al. (2006) and Lawrence et al. (1994). To establish such a real time
 233 PCR assay for diagnostic purposes, validation against blinded samples is required. Sensitivity and
 234 specificity for the assay should be determined against each target. Support for development of assays
 235 and appropriate sample panels can be obtained from the WOAH Reference Laboratories. Reference
 236 material and sample panels for real-time PCR can be obtained from the WOAH Reference Laboratories.

237 • Point of care (POC) molecular tests

238 Loop-mediated isothermal amplification (LAMP) assays for the detection of EHV-1
 239 have been described (Nemoto et al., 2011). An evaluation of a hydrolysis probe-
 240 based insulated isothermal PCR (iiPCR) assay for the detection of EHV-1 showed
 241 it to have a high sensitivity and specificity compared with real-time PCR (Balasuriya
 242 et al., 2017). However further validation of POC tests in the field is required.

243 • Molecular characterisation

244 Allelic discrimination real-time PCR assays identifying a single nucleotide
 245 polymorphism that was originally suggested to distinguish between
 246 neuropathogenic and non-neuro-pathogenic EHV-1 strains have been developed
 247 (Smith et al., 2012). However, investigations in many countries worldwide
 248 demonstrated that the nucleotide substitution was not a reliable predictor of
 249 enhanced neuropathogenicity. Multilocus typing and whole genome sequencing
 250 are useful for molecular epidemiological studies (Garvey et al., 2019; Nugent et al.,
 251 2006; Sutton et al., 2019).

252 1.3. Virus isolation

253 Virus isolation is no longer a routine test used for EHV-1 detection in the majority of diagnostic
 254 laboratories but is more often conducted for surveillance and research purposes. A number of
 255 cell types may be used for isolation of EHV-1 (e.g. rabbit kidney [RK-13 (AATC-CCL37)], baby
 256 hamster kidney [BHK-21], Madin-Darby bovine kidney [MDBK], pig kidney [PK-15], etc.).

257 RK13 cells are commonly used for this purpose. For efficient primary isolation of EHV-4 from
258 horses with respiratory disease, equine derived cell cultures must be used. Both EHV-1 and
259 EHV-4 may be isolated from nasopharyngeal samples using primary equine fetal kidney cells
260 or equine fibroblasts derived from dermal (E-Derm) or lung tissue. EHV-1 can be isolated on
261 other cell types, as will be discussed later. The nasopharyngeal swab and its accompanying
262 transport medium are transferred into the barrel of a sterile 10 ml syringe. Using the syringe
263 plunger, the fluid is squeezed from the swab into a sterile tube. A portion of the expressed fluid
264 can be filtered through a sterile, 0.45 µm membrane syringe filter unit into a second sterile
265 tube if heavy bacterial contamination is expected, but this may also lower virus titre. Recently
266 prepared cell monolayers in tissue culture flasks are inoculated with the filtered, as well as the
267 unfiltered, nasopharyngeal swab extract. Cell monolayers in multiwell plates incubated in a
268 5% CO₂ environment may also be used. Virus is allowed to attach by incubating the inoculated
269 monolayers at 37°C. Monolayers of uninoculated control cells should be incubated in parallel.

270 At Recently prepared cell monolayers in tissue culture flasks or plates are inoculated with
271 nasopharyngeal swab extract or homogenised tissue: approximately 10% (w/v) pooled tissue
272 homogenates of liver, lung, thymus, adrenal gland and spleen (from aborted fetuses/neonatal
273 foals) or of brain and spinal cord (from cases of neurological disease). Virus is allowed to
274 attach by incubating the end of the attachment period, inoculated monolayers at 37°C for 1
275 hour after which the inocula are removed and the monolayers are rinsed twice with PBS to
276 remove virus neutralising antibody that may or maintenance medium. Monolayers of
277 uninoculated control cells should be present in the nasopharyngeal secretions incubated in
278 parallel. After addition of supplemented maintenance medium (MEM containing 2% fetal calf
279 serum [FCS] and twice the standard concentrations of antibiotics/antifungals [penicillin,
280 streptomycin, gentamicin, and amphotericin B]), the flasks are incubated at 37°C in a 5% CO₂
281 environment.

282 The use of a positive control virus samples of relatively low titre may be used to validate the
283 isolation procedure carries the risk that this may lead but should be processed separately to
284 eventual avoid contamination of diagnostic specimens. This risk can be minimised by using
285 routine precautions and good laboratory technique, including the use of biosafety cabinets,
286 inoculating positive controls after the diagnostic specimens, decontaminating the surfaces in
287 the hood while the inoculum is adsorbing and using a positive control of relatively low titre.
288 Inoculated flasks should be inspected daily by microscopy for the appearance of characteristic
289 herpesvirus cytopathic effect (CPE) (focal rounding, increase in refractility, and detachment of
290 cells). Cultures exhibiting no evidence of viral CPE after 1 week of incubation should be blind-
291 passaged into freshly prepared monolayers of cells, using small aliquots of both media and
292 cells as the inoculum. Further blind passage is usually not productive.

293 It can be useful to inoculate samples into both non-equine and equine cells in parallel to
294 distinguish between EHV-1 and EHV-4, since EHV-4 can cause sporadic cases of abortion.
295 Around 10% (w/v) pooled tissue homogenates of liver, lung, thymus, and spleen (from aborted
296 fetuses) or of central nervous system tissue (from cases of neurological disease) are used for
297 virus isolation. These are prepared by first mincing small samples of tissue into 1 mm cubes
298 in a sterile Petri dish with dissecting scissors, followed by macerating the tissue cubes further
299 in serum free culture medium with antibiotics using a homogeniser or mechanical tissue
300 grinder. After centrifugation at 1200 g for 10 minutes, the supernatant is removed and 0.5 ml
301 is inoculated into duplicate cell monolayers in tissue culture flasks. Following incubation of the
302 inoculated cells at 37°C for 1.5–2 hours, the inocula are removed and the monolayers are
303 rinsed twice with PBS or maintenance medium. After addition of 5 ml of supplemented
304 maintenance medium, the flasks are incubated at 37°C for up to 1 week or until viral CPE is
305 observed. Cultures exhibiting no evidence of viral CPE after 1 week of incubation should be
306 passaged a second time into freshly prepared monolayers of cells, using small aliquots of both
307 media and cells as the inoculum.

308 Blood samples: EHV-1 and, infrequently, EHV-4 can be isolated from PBMC. Buffy coats may
309 be prepared from unclotted (heparinised) blood by centrifugation at 600–525 g for 15
310 minutes, and. The buffy coat is taken after the plasma has been carefully removed. The buffy
311 coat is then layered onto a PBMC separating solution (Ficoll; density 1077 g/ml, commercially
312 available) and centrifuged at 400 g for 20 minutes. The PBMC interface (without most

313 granulocytes) is and washed twice in PBS (300 g for 10 minutes) and resuspended in 1 ml
314 three times in 3 ml MEM containing 2% FCS. As a quicker alternative method, PBMC may be
315 collected by centrifugation directly from plasma. (525 g for 5 minutes). Following the third
316 wash, the buffy coat is harvested and resuspended in 2.5 ml MEM containing 2% FCS. An
317 aliquot of the rinsed cell suspension is added to each of the duplicate monolayers of equine
318 fibroblast, equine fetal or RK-13 cell monolayers in 25 cm² flasks containing 8–10 ml freshly
319 added maintenance medium. The flasks can be used for DNA extraction. For virus isolation,
320 the resuspended cells (1 ml) are co-cultivated with freshly prepared primary equine lung or
321 RK-13 cell suspensions (5 ml) in 25 cm² flasks. Confluent cell monolayers are not used. The
322 flasks are incubated at 37°C in a 5% CO₂ environment for 3 days or until the cells have
323 reached 90% confluence. The monolayers are then rinsed three times with 1 × PBS and
324 supplemented with 5 ml MEM containing 2% FCS. They are incubated at 37°C for 7 days;
325 either with or without removal of the inoculum. If PBMCs are not removed prior to incubation,
326 CPE may be difficult to detect in the presence of the massive inoculum of leukocytes: each
327 flask of cells is freeze-thawed after 7 for a further 4 days of incubation and the contents
328 centrifuged at 300 g for 10 minutes. Finally, 0.5 ml of the cell-free, culture medium supernatant
329 is transferred to freshly made cell monolayers that are just subconfluent. These are incubated
330 and observed daily for viral CPE for at least 5–6 days. Again, samples exhibiting no
331 evidence of viral CPE after 1 week of incubation should be passaged a second time before
332 discarding as negative.

333 Virus identity may be confirmed by PCR or by immunofluorescence with specific antisera.
334 Virus isolates from positive cultures should be submitted to a WOAHP Reference Laboratory
335 for strain characterisation and to maintain a geographically diverse archive. Further strain
336 characterisation for surveillance purposes or detection of the neurological marker can be
337 provided at some laboratories.

338 **1.4. Virus detection by direct immunofluorescence**

339 Direct immunofluorescent detection of EHV-1 antigens in samples of post-mortem tissues
340 collected from aborted equine fetuses and the placenta provides a rapid preliminary diagnosis
341 of herpesvirus abortion (Gunn, 1992). The diagnostic reliability of this technique approaches
342 that of virus isolation attempts from the same tissues.

343 In the United States of America (USA), potent polyclonal antiserum to EHV-1, prepared in
344 swine and conjugated with FITC, is available to veterinary diagnostic laboratories for this
345 purpose from the National Veterinary Services Laboratories of the United States Department
346 of Agriculture (USDA). The antiserum cross-reacts with EHV-4 and hence is not useful for
347 serotyping, however virus typing can be conducted on any virus positive specimens by PCR.

348 Freshly dissected samples (5 × 5 mm pieces) of fetal tissue (lung, liver, thymus, and spleen)
349 are frozen, sectioned on a cryostat at –20°C, mounted on to microscope slides, and fixed with
350 100% acetone. After air-drying, the sections are incubated at 37°C in a humid atmosphere for
351 30 minutes with an appropriate dilution of the conjugated swine antibody to EHV-1. Unreacted
352 antibody is removed by two washes in PBS, and the tissue sections are then covered with
353 aqueous mounting medium and a cover-slip, and examined for fluorescent cells indicating the
354 presence of EHV antigen. Each test should include a positive and negative control consisting
355 of sections from known EHV-1 infected and uninfected fetal tissue.

356 **1.5. Virus detection by immunoperoxidase staining**

357 Immunohistochemical (IH) staining methods, such as immunoperoxidase, have been
358 developed for detecting EHV-1 antigen in fixed tissues of aborted equine fetuses, placental
359 tissues or neurologically affected horses (Schultheiss *et al.*, 1993; Whitwell *et al.*, 1992). Such
360 techniques can be used as an alternative to immunofluorescence described above and can
361 also be readily applied to archival frozen or fixed tissue samples. Immunohistochemical
362 staining for EHV-1 is particularly useful for the simultaneous evaluation of morphological
363 lesions and the identification of the virus. Immunoperoxidase staining for EHV-1/4 may also
364 be carried out on infected cell monolayers (van Maanen *et al.*, 2000). Adequate controls must
365 be included with each immunoperoxidase test run for evaluation of both the method specificity

366 and antibody specificity. ~~In one WOAH Reference Laboratory, this method is used routinely~~
367 ~~for frozen or fixed tissue, using If non-specific rabbit polyclonal sera is used raised against~~
368 ~~EHV-1. This staining method is not type specific and therefore the staining method needs to~~
369 ~~be combined with virus isolation or PCR to discriminate between EHV-1 and EHV-4, however~~
370 ~~it provides a useful method for rapid diagnosis of EHV-induced abortion.~~

371 1.6. Histopathology

372 Histopathological examination of sections of fixed placenta and lung, liver, spleen, adrenal
373 gland and thymus from aborted fetuses and brain and spinal cord from neurologically affected
374 horses should be carried out. In aborted fetuses, eosinophilic intranuclear inclusion bodies
375 present within bronchiolar epithelium or in cells at the periphery of areas of hepatic necrosis
376 are consistent with a diagnosis of herpesvirus infection. The characteristic microscopic lesion
377 associated with EHV-1 neuropathy is a degenerative thrombotic vasculitis of small blood
378 vessels in the brain or spinal cord (perivascular cuffing and infiltration by inflammatory cells,
379 endothelial proliferation and necrosis, and thrombus formation).

380 2. Serological tests

381 EHV-1 and EHV-4 are endemic in most parts of the world and seroprevalence is high, however
382 serological testing of paired sera can be useful for diagnosis of ER in horses. A positive diagnosis is
383 based on the demonstration of significant increases (four-fold or greater) in antibody titres in paired sera
384 taken during the acute and convalescent stages of the disease. The results of tests performed on sera
385 from a single collection date are, in most cases, impossible to interpret with any degree of confidence.
386 The initial (acute phase) serum sample should be taken as soon as possible after the onset of clinical
387 signs, and the second (convalescent phase) serum sample should be taken 2–4 weeks later.

388 'Acute phase' sera from mares after abortion or from horses with EHV-1 neurological disease may
389 already contain maximal titres of EHV-1 antibody, with no increase in titres detectable in sera collected
390 at later dates. In such cases, serological testing of paired serum samples from clinically unaffected
391 cohort members of the herd may prove useful for retrospective diagnosis of ER within the herd.

392 Finally, the serological detection of antibodies to EHV-1 in heart or umbilical cord blood or other fluids
393 of equine fetuses can be of diagnostic value in cases of abortion especially when the fetus is virologically
394 negative. The EHV 1/4 nucleic acid may be identified from these tissues by PCR.

395 Serum antibody levels to EHV-1/4 may be determined by virus neutralisation (VN) (Thomson *et al.*,
396 1976), complement fixation tests (CFT) (Thomson *et al.*, 1976) or enzyme-linked immunosorbent assay
397 (ELISA) (Crabb & Studdert, 1995). ~~There are no internationally recognised reagents or standardised~~
398 ~~techniques for performing any of the serological tests for detection of EHV-1/4 antibody; titre~~
399 ~~determinations on the same serum may differ from one laboratory to another. Furthermore, The CF and~~
400 ~~VN tests detect antibodies that are cross-reactive between EHV-1 and EHV-4. Nonetheless, the~~
401 ~~demonstration of a four-fold or greater rise in antibody titre to EHV-1 or EHV-4 during the course of a~~
402 ~~clinical illness provides serological confirmation of recent infection with one of the viruses. Commercial~~
403 ~~ELISAs that distinguish EHV-1 and EHV-4 antibodies are available but less widely used than the CF~~
404 ~~and VN tests. Unlike other alphaherpesviruses, DIVA ELISAs, which have been very useful in~~
405 ~~eradication programmes for bovine rhinotracheitis and pseudorabies (Aujeszky's disease), have not~~
406 ~~been developed for EHV-1/4. An ELISA using a synthetic peptide for glycoprotein E as an antigen~~
407 ~~(Andoh *et al.*, 2013) is used as DIVA¹ for horses vaccinated with a modified live EHV-1 vaccine licensed~~
408 ~~in Japan, that lacks the glycoprotein E gene.~~

409 ~~The microneutralisation test is a VN and the CF tests are~~ widely used and sensitive serological assays
410 for detecting EHV-1/4 antibody and will thus be described here.

411 2.1. Virus neutralisation test

412 This test is most commonly performed in flat-bottom 96-well microtitre plates (tissue culture
413 grade) using a constant dose of virus and doubling dilutions of equine test sera. At least two

¹ DIVA: detection of infection in vaccinated animals

414 three replicate wells for each serum dilution are required. Heat-inactivated maintenance
415 medium with a concentration of 2% FCS (HIMM) Serum-free MEM is used throughout as a
416 diluent. Virus stocks of known titre are diluted just before use to contain 100 TCID₅₀ (50%
417 tissue culture infective dose) in 25 µl. Monolayers of E-Derm or RK-13 cells are prepared
418 monodispersed with EDTA/trypsin and resuspended at a concentration of 5 × 10⁵/ml. Note
419 that RK-13 cells can be used with EHV-1 but do not show CPE with EHV-4. Antibody positive
420 and negative control equine sera and controls for cell viability, virus infectivity, and test serum
421 cytotoxicity, must be included in each assay. End-point VN titres of antibody are calculated by
422 determining the reciprocal of the highest serum dilution that protects $\geq 75\%$ 100% of the cell
423 monolayer from virus destruction in both of the replicate wells.

424 Serum toxicity may be encountered in samples from horses repeatedly vaccinated with a
425 commercial vaccine prepared from EHV-1 grown up in RK-13 cells. This can give rise to
426 difficulties in interpretation of test reactions at lower serum dilutions. The problem can be
427 overcome using E-Derm or other non-rabbit kidney derived cell line.

428 **2.1.1. Test procedure**

429 A suitable test procedure is as follows:

- 430 i) Prepare semi-confluent monolayers in tissue culture microtitre plates.
 - 431 ii) Inactivate test and control sera for 30 minutes in a water bath at 56°C.
 - 432 iii) Add 40 25-µl of HIMM serum free MEM to all wells of the microtitre assay plates.
 - 433 iv) For test sample titration, pipette 25-40 µl of each test serum into duplicate triplicate
434 wells of both rows A and B of the plate. The first two rows serve as the dilution of
435 the test serum and the third row serves as the serum toxicity control and the second
436 row as the first dilution of the test. Make doubling dilutions of each serum starting
437 with row B and proceeding to the bottom of the plate by sequential mixing and
438 transfer of 25-40 µl to each subsequent row of wells. Six sera can be assayed in
439 each plate. Add 40µl of HIMM to the serum control rows.
 - 440 v) Add 40 25-µl of the appropriately diluted EHV-1 or EHV-4 virus stock to each all
441 wells (100 TCID₅₀/well) of the test plate except those of row A, which are the serum
442 controls wells. Note that the final serum dilutions, after addition of virus, run from a
443 starting dilution of 1/4 to 1/256. A separate control plate should include titration of
444 both a negative and positive (high and low) horse serum sera of known titre, cell
445 control (no virus), and a back titration of virus control (no serum), and a virus
446 titration using six wells per log dilution (100 TCID₅₀ to 0.01 TCID₅₀/well) calculate
447 the actual amount of virus used in the test
 - 448 vi) Incubate the plates for 1 hour at 37°C in 5% CO₂ atmosphere. Add 50 µl of the
449 prepared E-Derm or RK-13 cell suspension (5 × 10⁵ cells/ml) in MEM/10% FCS to
450 each well.
 - 451 vii) Transfer 50 µl from each well of the test and control plates to the tissue culture
452 microtitre plates.
 - 453 viii) Incubate the plates for 2-4-5 days at 37°C in an atmosphere of 5% CO₂ in air.
 - 454 ix) Examine the plates microscopically for CPE and record the results on a worksheet.
455 Confirm the validity of the test by establishing that the working dilution of stock virus
456 is at 100 TCID₅₀/well, that the (high and low) positive control sera are within one
457 well of their pre-determined titre and that the negative control serum is negative at
458 a 1/4 dilution. This takes approximately 72 hours. If at this stage the antigen is too
459 weak the virus concentration may be increased by extending the incubation period
460 up to 5 days. If the antigen is too strong the test must be repeated.
- 461 Wells are scored as positive for neutralisation of virus if $\geq 75\%$ of the cell monolayer
462 remains intact. The highest dilution of serum resulting in $\geq 75\%$ neutralisation of
463 virus (<25% CPE) in replicate wells is the end-point titre for that serum. Examine
464 the plates microscopically for CPE and record the results on a worksheet.

- 465 x) Alternatively, the cell monolayers can be scored for CPE after fixing and staining
466 as follows: after removal of the culture fluid, immerse the plates for 15 minutes in
467 a solution containing 2 mg/ml crystal violet, 10% formalin, 45% methanol, and 45%
468 water. Then, rinse the plates vigorously under a stream of running tap water. Wells
469 containing intact cell monolayers stain blue, while monolayers destroyed by virus
470 do not stain. ~~Verify that the cell control, positive serum control, and serum
471 cytotoxicity control wells stain blue, that the virus control and negative serum
472 control wells are not stained, and that the actual amount of virus added to each
473 well is between $10^{4.5}$ and $10^{2.5}$ TCID₅₀. Wells are scored as positive for
474 neutralisation of virus if 100% of the cell monolayer remains intact. The highest
475 dilution of serum resulting in complete neutralisation of virus (no CPE) in both
476 duplicate wells is the end-point titre for that serum.~~
- 477 xi) Calculate the neutralisation titre for each test serum, and compare acute and
478 convalescent phase serum titres from each animal for a four-fold or greater
479 increase.

480 **2.2. Complement fixation test**

481 The CFT can be used for the detection and quantification of antibodies against to EHV-1. The
482 test determines whether an antigen and an antibody are capable of forming a complex. The
483 presence of an immune complex is revealed by the detector system, which consists of guinea-
484 pig complement and sensitised sheep red blood cells (SRBCs) coated with rabbit haemolytic
485 serum (haemolysin). In the absence of antibodies against equine herpesvirus, no
486 antibody/antigen complex is formed, the complement remains free in the solution and the
487 sensitised SRBCs become lysed. In the presence of antibodies against equine herpesvirus,
488 an antibody/antigen complex is formed, the complement becomes fixed and is therefore
489 unable to lyse the SRBCs. They subsequently form a button at the bottom of the test well.

490 Guinea-pig complement, rabbit haemolytic serum, complement fixation diluent (CFD) and
491 bovine serum albumin (BSA) can be obtained commercially. The dilution of guinea-pig
492 complement that has activity at 3 HD (haemolytic dose) in the presence of sensitised SRBCs
493 should be optimised. The recommended dilution of rabbit haemolytic serum (or the working
494 dilution) is sometimes provided by the supplier. However, the optimal dilution of haemolysin
495 should be determined with the in use reagents (complement etc.) so that the test can be
496 performed reproducibly. The optimum concentration of antigen to be used in the test should
497 be determined using an antigen versus antibody chequerboard technique and by testing a
498 panel of known positive sera.

499 The test is performed in U bottomed microtitre plates. Paired sera should be assayed on the
500 same plate. An antibody positive serum should be included as a control on each plate. All sera
501 are tested on a second plate containing all components except virus to check for anti-
502 complementary activity. A back titration of the working dilution (3 HD) of complement to 2 HD,
503 1 HD, 0.5 HD is set up in duplicate wells on the complement control plate (eight wells in total).
504 An SRBC control is set up in eight wells.

505 **2.2.3. Preparation of samples**

- 506 i) Samples and controls are prepared by adding 4 volumes (600 µl) of CFD to 1
507 volume (150 µl) of test sera to give a 1/5 dilution.
- 508 ii) Diluted serum is inactivated for 30 minutes at 60°C to destroy the naturally
509 occurring complement.

510 **2.2.4. Test procedure**

- 511 i) Prepare the test plate and anti-complementary plate by adding 25 µl 0.05%
512 BSA/CFD to all wells except the first column (H).
- 513 ii) Add 50 µl of 0.05% BSA/CFD to the eight wells of the complement control (back
514 titration).
- 515 iii) Add 75 µl of 0.05% BSA/CFD to eight wells of cell control.

- 516 iv) Add 50 µl of the diluted inactivated test serum and controls to the first well of each
517 row on both the test and anti-complementary plates. Serial doubling dilutions are
518 then made by transferring 25 µl across the plate and discarding the final 25 ml.
- 519 v) Place the microtitre plates on ice for addition of antigen and complement.
- 520 vi) Add 25 µl of antigen (diluted to working strength in 0.05% BSA/CFD) to the test
521 plates.
- 522 vii) Add 25 µl of 0.05% BSA/CFD to all wells of the anti-complementary plate to
523 compensate for lack of antigen.
- 524 viii) Add 25 µl of guinea-pig complement diluted in 0.05% BSA/CFD to 3 HD to all wells
525 except the complement control and SRBC control.
- 526 ix) Back titrate the working dilution of 3 HD complement to 2 HD, 1 HD and 0.5 HD in
527 200 µl volumes. Add 25 µl of each dilution to the appropriate wells.
- 528 x) Incubate all plates at 4°C overnight.

529 **2.2.5. Preparation and addition of sheep blood**

- 530 i) SRBCs collected into Alsever's solution are washed twice in 0.05% BSA/PBS
531 solution.
- 532 ii) Gently resuspend the SRBCs in 5–10 ml 0.05% BSA/CFD solution. Dilute to 2%
533 SRBCs (v/v packed cells) in BSA/CFD solution.
- 534 iii) Mix the 2% SRBCs with an equal volume of BSA/CFD solution containing
535 haemolysin at its optimal sensitising concentration to give a 1% SRBC solution.
536 Prepare an appropriate volume of this solution by allowing 3 ml per microtitre plate.
- 537 iv) Incubate at 37°C for 10 minutes. Store the 1% sensitised SRBCs overnight at 4°C.
- 538 v) The following day, incubate the 1% sensitised SRBCs at 37°C for 30 minutes.
539 During the final 20 minutes of this incubation, transfer the test plates from 4°C to
540 37°C.
- 541 vi) At the end of the 30-minute incubation, add 25 ml of 1% sensitised SRBCs to all
542 plates. Mix on a plate shaker for 30 seconds.
- 543 vii) Incubate the plates at 37°C for 30 minutes. Shake the plates after 15 minutes and
544 at the end of this incubation (a total of three times).
- 545 viii) Incubate the plates at 4°C for 2 hours to allow the cells to settle.
- 546 ix) Read and record the test results after 2 hours.

547 **2.2.6. Reading results**

- 548 i) Confirm the validity of the test by establishing that the working dilution of
549 complement is at 3 HD: 100% lysis at 3 HD and 2 HD, and 50% lysis at 1 HD.
550 Distinct buttons should be visible in the eight wells of the SRBC control.
- 551 ii) There must be 100% lysis observed at the 1/5 dilution for the negative control (<5).
552 The antibody titre of the positive control serum must read within one well of its
553 predetermined titre.
- 554 iii) Confirm that there are no buttons visible on the anti-complementary plates.
555 Buttoning indicates either the presence of residual native complement in the
556 sample or that there is a non-specific complement fixing effect occurring. Sera that
557 show anti-complementary activity should be retested and treated as described
558 below.
- 559 iv) In the test wells, buttoning indicates the presence of antibodies in the serum. The
560 antibody titre is the dilution at which there is 50% buttoning and 50% lysis
561 observed.

562

2.2.7. Treatment of samples showing anti-complementary activity

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i) Add 50 µl of guinea-pig complement to 150 µl of the serum showing anti-complementary activity.

564

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ii) Incubate the sample at 37°C for 30 minutes.

566

iii) Add 550 µl of CFD (1:5 dilution).

567

iv) Heat inactivate at 60°C for 30 minutes.

568

C. REQUIREMENTS FOR VACCINES

569

1. Background

570

Both live attenuated and inactivated vaccines are available for use in horses as licensed, commercially prepared products for use in reducing the impact of disease in horses caused by EHV-1/4 infection. The products contain different permutations of EHV-1 and EHV-4 and some also include equine influenza virus.

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Clinical experience has demonstrated that vaccination can be useful for reducing clinical signs of respiratory disease and incidence of abortion, however none of the vaccines protect against neurological disease. Multiple doses repeated annually, of each of the currently marketed ER vaccines are recommended by their respective manufacturers. Vaccination schedules vary with a particular vaccine.

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The indications stated on the product label for use of several available vaccines for ER are either as a preventative of herpesvirus-associated respiratory disease, or as an aid in the prevention of abortion, or both. A minority of ~~Only four~~ vaccine products have met the regulatory requirements for claiming efficacy in providing protection from herpesvirus abortion as a result of successful vaccination and challenge experiments in pregnant mares. None of the vaccine products have been demonstrated to prevent the occurrence of neurological disease sometimes associated with EHV-1 infection.

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Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

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587

2. Outline of production and minimum requirements for vaccines

588

2.1. Characteristics of the seed

589

The master seed virus (MSV) for ER vaccines must be prepared from strains of EHV-1 and/or EHV-4 that have been positively and unequivocally identified ~~by both serological and genetic tests~~. Seed virus must be propagated in a cell line approved for equine vaccine production by the appropriate regulatory agency. A complete record of original source (including isolate number, location, year of isolation), passage history, medium used for propagation, etc., shall be kept for the master seed preparations of both the virus(es) and cell stock(s) intended for use in vaccine production.

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2.1.1. Biological characteristics of the master seed

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Permanently stored stocks of both MSV and master cell stock (MCS) used for vaccine production must be demonstrated to be pure, safe and, in the case of MSV, also immunogenic.

598

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Generally, the fifth passage from the MSV and the twentieth passage from the MCS are the highest allowed for vaccine production. Results of all quality control tests on master seeds must be recorded and made a part of the licensee's permanent records.

601

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603 **2.1.2. Quality criteria**

604 Tests for master seed purity include prescribed procedures that demonstrate the virus
605 and cell seed stocks to be free from bacteria, fungi, mycoplasmas, and extraneous
606 viruses. Special tests must be performed to confirm the absence of equine arteritis virus,
607 equine infectious anaemia virus, equine influenza virus, equine herpesvirus-2, -3, and -
608 5, equine rhinitis A and B viruses, the alphaviruses of equine encephalomyelitis, bovine
609 viral diarrhoea virus (BVDV – common contaminant of bovine serum), and porcine
610 parvovirus (PPV – potential contaminant of porcine trypsin). The purity check should
611 also include the exclusion of the presence of EHV-1 from EHV-4 MSV and *vice versa*.

612 **2.1.3. Validation as a vaccine strain**

613 Tests for immunogenicity of the EHV-1/4 MSV stocks should be performed in horses on
614 an experimental test vaccine prepared from the highest passage level of the MSV
615 allowed for use in vaccine production. The test for MSV immunogenicity consists of
616 vaccination of horses with low antibody titres (< 1:24 by VN test) to EHV-1/4, with doses
617 of the test vaccine that will be recommended on the final product label (Goodman *et al.*,
618 2006; Van de Walle *et al.*, 2010). Second serum samples should be obtained and tested
619 for significant increases in neutralising antibody titre against the virus, 21 days after the
620 final dose.

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

630 **2.2. Method of manufacture**

631 **2.2.1. Procedure**

632 A detailed protocol of the methods of manufacture to be followed in the preparation of
633 vaccines for ER must be compiled, approved, and filed as an Outline of Production with
634 the appropriate licensing agency. Specifics of the methods of manufacture for ER
635 vaccines will differ with the type (live or inactivated) and composition (EHV-1 only, EHV-
636 1 and EHV-4, EHV-4 and equine influenza viruses, etc.) of each individual product, and
637 also with the manufacturer.

638 **2.2.2. Requirements for ingredients**

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

643 **2.2.3. Final product batch tests**

644 i) Sterility

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

- 651 ii) Identity
- 652 Identity tests shall demonstrate that no other vaccine strain is present when several
- 653 strains are propagated in a laboratory used in the production of multivalent
- 654 vaccines.
- 655 iii) Safety
- 656 Safety tests shall consist of detecting any abnormal local or systemic adverse
- 657 reactions to the vaccine in the host species by all vaccination route(s). Tests to
- 658 assure safety of each production batch of ER vaccine must demonstrate complete
- 659 inactivation of virus (for inactivated vaccines) as well as a level of residual virus-
- 660 killing agent that does not exceed the maximal allowable limit (e.g. 0.2% for
- 661 formaldehyde).
- 662 iv) Batch potency
- 663 Batch potency is examined on the final formulated product. ~~Batch control of~~
- 664 ~~antigenic potency for EHV-1 vaccines only may be tested by measuring the ability~~
- 665 ~~of dilutions of the vaccine to protect hamsters from challenge with a lethal dose of~~
- 666 ~~hamster adapted EHV-1 virus. Although potency testing on production batches of~~
- 667 ~~ER vaccine may also be performed by vaccination of susceptible horses followed~~
- 668 ~~by assay for seroconversion, the recent availability of virus type specific MABs has~~
- 669 ~~permitted development of less costly and more rapid *in-vitro* immunoassays exist~~
- 670 ~~for antigenic potency. The basis for such *in-vitro* assays for ER vaccine potency is~~
- 671 ~~the determination, by use of the specific MAB, of the presence of at least the~~
- 672 ~~minimal amount of viral antigen within each batch of vaccine that correlates with~~
- 673 ~~the required level of protection (or seroconversion rate) in a standard animal test~~
- 674 ~~for potency.~~

675 2.3. Requirements for authorisation/registration/licencing

676 2.3.1. Manufacturing process

677 For registration of vaccine, all relevant details concerning manufacture of the vaccine

678 and quality control testing (see Sections C.2.1 and C.2.2) should be submitted to the

679 authorities. This information shall be provided from three consecutive vaccine batches

680 with a volume not less than 1/3 of the typical industrial batch volume.

681 2.3.2 Safety requirements

682 Vaccine safety should be evaluated in vaccinated animals using different assays (see

683 Section 2.2.3.iii).

684 2.3.3 Efficacy requirements

685 Vaccine efficacy (protection) is estimated in vaccinated animals directly by evaluating

686 their resistance to live pathogen challenge.

687 2.3.4 Duration of immunity

688 As part of the licensing or marketing authorisation procedure, the manufacturer may be

689 required to demonstrate the duration of immunity (DOI) of a given vaccine by either

690 challenge or alternative test at the end of the claimed period of protection.

691 Tests to establish the duration of immunity to EHV-1/4 ~~or EHV1/4~~ achieved by

692 immunisation with each batch of vaccine are not required. The results of many reported

693 observations indicate that immunity induced by vaccination against EHV-1 or EHV

694 ~~induced immunity to EHV 1/4~~ is not more than a few months in duration; these

695 observations are reflected in the frequency of revaccination recommended on ER

696 vaccine product labels.

697 **2.3.5 Stability**

698 As part of the licensing or marketing authorisation procedure, the manufacturer will be
699 required to demonstrate the stability of all the vaccine's properties at the end of the
700 claimed shelf-life period. Storage temperature shall be indicated, and warnings should
701 be given if product is damaged by freezing or ambient temperature.

702 At least three production batches of vaccine should be tested for shelf life before
703 reaching a conclusion on the vaccine's stability. When stored at 4°C, inactivated vaccine
704 products generally maintain their original antigenic potency for at least 1 year.
705 Lyophilised preparations of the live virus vaccine are also stable during storage for 1
706 year at 4°C. Following reconstitution, live virus vaccine is unstable and cannot be stored
707 without loss of potency.

708 **Note:** current vaccines are authorised for prevention of respiratory disease or as an aid in the prevention
709 of abortion. Unless the vaccine's ability to prevent neurological disease is under investigation, the virus
710 used in the challenge experiments should not be a strain with a history of inducing neurological disease.

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888 *
889 * *

890 **NB:** There are WOAHP Reference Laboratories for equine rhinopneumonitis (please consult the WOAHP
891 Web site:

892 <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>).

893 Please contact the WOAHP Reference Laboratories for any further information on
894 diagnostic tests, reagents and vaccines for equine rhinopneumonitis
895 and to submit strains for further characterisation.

896 **NB:** FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2017.

Annexe 14. Chapter 3.8.1. 'Border disease'

SECTION 3.8.

~~OVIDAE AND CAPRINAE~~

CHAPTER 3.8.1.

BORDER DISEASE

SUMMARY

Border disease (BD) is a viral disease of sheep and goats first reported in sheep in 1959 from the border region of England and Wales, and since recorded world-wide. Prevalence rates in sheep vary from 5% to 50% between countries and from region to region within countries. Clinical signs include barren ewes, abortions, stillbirths and the birth of small weak lambs. Affected lambs can show ~~and a fine~~ tremor, abnormal body conformation and hairy fleeces (so-called 'hairy-shaker' or 'fuzzy' lambs). Consequently, the disease has sometimes been referred to as 'hairy shaker disease'. Vertical transmission plays an important role in the epidemiology of the disease. Infection of fetuses can result in the birth of persistently infected (PI) lambs. These PI lambs are viraemic, antibody negative and constantly excrete virus. The virus spreads from sheep to sheep, with PI animals being the most potent source of infection. Infection in goats is less common with abortion being the main presenting sign.

BD is caused by the Pestivirus border disease virus (BDV), but in some parts of the world, especially where there is close contact between sheep or goats and cattle, the same clinical signs may be caused by infection with bovine viral diarrhoea virus (BVDV). Therefore the genetic and antigenic differences between BDV and BVDV need to be taken into consideration when investigating disease outbreaks or certifying animals or germplasm for international movement. It is important to identify the viraemic PI animals so that they will not be used for breeding or trading purposes. Serological testing is insufficient. However, it is generally considered that serologically positive, nonviraemic sheep ~~are 'safe', do not present a risk~~ as latent infections are not known to occur in recovered animals. Pregnant seropositive, nonviraemic animals may, however, present a risk by carrying a PI fetus that cannot be detected until after parturition.

Identification of the agent: BDV is a species of Pestivirus (Pestivirus ovis) in the family Flaviviridae and is closely related to classical swine fever virus (Pestivirus suis) and BVDV viruses, which are classified in the distinct species: Pestivirus bovis (commonly known as BVDV type 1), Pestivirus tauri (formerly BVDV type 2) and Pestivirus brazilense (BVDV type 3 or Hobi-like pestivirus). Nearly all isolates of BDV are noncytopathogenic in cell culture. There are no defined serotypes but virus isolates exhibit considerable antigenic diversity. A number of separate genotypes have been identified.

Apparently healthy PI sheep resulting from congenital infection can be identified by direct detection of virus or nucleic acid in blood or tissues or by virus isolation in cell culture followed by immunostaining to detect the noncytopathogenic virus.

Diagnostic methods: The demonstration of virus by culture and antigen detection may be less reliable in lambs younger than 2 months that have received colostral antibody. Acute infection is usually subclinical and viraemia is transient and difficult to detect. The isolation of virus from

40 tissues of aborted or stillborn lambs is often difficult but virus can be detected by sensitive reverse
41 transcriptase polymerase chain reaction methods that are able to detect residual nucleic acid.
42 However, tissues and blood from PI sheep more than a few months old contain high levels of
43 virus, which can be easily identified by isolation and direct methods to detect antigens or nucleic
44 acids. As sheep may be infected with BVDV, it is preferable to use diagnostic assays that are
45 'pan-pestivirus' reactive and will readily detect all strains of BDV and BVDV.

46 **Serological tests:** Acute infection with BDV is best confirmed by demonstrating seroconversion
47 using paired or sequential samples from several animals in the group. The enzyme-linked
48 immunosorbent assay and virus neutralisation test (VNT) are the most commonly used antibody
49 detection methods. Due to the antigenic differences between BDV and BVDV, assays for the
50 detection of antibodies to BDV, especially by VNT, should preferably be based on a strain of BDV.

51 **Requirements for vaccines:** There is no standard vaccine for BDV, but a commercial killed
52 whole-virus vaccine has been produced. Ideally, such a vaccine should be suitable for
53 administration to females before breeding for prevention of transplacental infection. The use of
54 BVDV vaccines has been advocated, but the antigenic diversity of BD viruses must be
55 considered. In many instances, the antigenic diversity of BDV strains is sufficiently different to
56 BVDV that a BVDV vaccine is unlikely to provide protection.

57 BD viruses have contaminated several modified live veterinary vaccines produced in sheep cells
58 or containing sheep serum. This potential hazard should be recognised by manufacturers of
59 biological products.

60 A. INTRODUCTION

61 Border disease virus (BDV) is a *Pestivirus* of the family *Flaviviridae* and is closely related to classical swine
62 fever virus (CSFV) and bovine viral diarrhoea virus (BVDV). There are ~~four~~ a number of officially recognised
63 species, namely – BDV (*Pestivirus ovis*) CSFV (*Pestivirus suis*), BVDV types 1 and 2 (taxonomically known
64 as *Pestivirus bovis* and *Pestivirus tauri*, respectively) and BDV (ICTV, 2016)-BVDV 3 or Hobi-like pestivirus
65 (*Pestivirus brazilense*) (Postler et al., 2023), but a number of other pestiviruses that are considered to be
66 distinct species have been reported. While CSF viruses are predominantly restricted to pigs, examples of there
67 are situations where the other ~~three~~ species have all been recovered from sheep. While the majority of isolates
68 have been identified as BD viruses in areas where sheep or goats are raised in isolation from other species
69 (Vilcek et al., 1997), in regions where there is close contact between small ruminants and cattle, BVDV may
70 be frequently identified (Carlsson, 1991). Nearly all virus isolates of BDV are noncytopathogenic, although
71 occasional cytopathic viruses have been isolated (Vantsis et al., 1976). BDV spreads naturally among sheep
72 by the oro-nasal route and by vertical transmission. It is principally a cause of congenital disease in sheep and
73 goats, but can also cause acute and persistent infections. Infection is less common in goats, in which persistent
74 infection is rare as abortion is the main presenting sign. Pigs may also be infected by pestiviruses other than
75 CSFV and antibodies to BDV in pigs may interfere with tests for the diagnosis of CSF (Oguzoglu et al., 2001).
76 Several genotypes of BD viruses from sheep, goats and Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*)
77 have been described. Phylogenetic analysis using computer-assisted nucleotide sequence analysis suggests
78 that genetic variability among BD viruses is greater than within each of the other *Pestivirus* species. Four
79 distinguishable genogroups of BDV have been described as well as putative novel *Pestivirus* genotypes from
80 Tunisian sheep and a goat At least eight BDV genotypes have been described (BDV type 1 to BDV type 8).
81 Other ovine pestiviruses have been identified that are responsible for BD-like syndromes such as Tunisian and
82 Tunisian-like, Aydin-like (*Pestivirus* I, Turkey) *Pestivirus* genotypes from Tunisian sheep and a goat and a new
83 emerging ovine pestivirus (OVPV) that were found to be genetically and antigenically closely related to CSFV
84 (Becher et al., 2003; Righi et al., 2021; Vilcek & Nettleton, 2006). The chamois BDV is similar to isolates from
85 sheep in the Iberian Peninsula (Valdazo-Gonzalez et al., 2007). This chapter describes BDV infection in sheep.
86 Chapter 3.4.7 *Bovine viral diarrhoea* should also be consulted for related diagnostic methods.

87 1. Acute infections

88 Healthy newborn and adult sheep exposed to BDV usually experience only mild or inapparent disease. Slight fever and a
89 mild leukopenia are associated with a short-lived viraemia detectable between days 4 and 11 post-infection,
90 after which virus neutralising antibody appears in the serum (Thabti et al., 2002).

91 Acute infections are best diagnosed serologically using paired sera from a representative number of sheep.
92 Occasional BDV isolates have been shown to produce high fever, profound and prolonged leukopenia,
93 anorexia, conjunctivitis, nasal discharge, dyspnoea and diarrhoea, and 50% mortality in young lambs. One
94 such isolate was recovered from a severe epidemic of BD among dairy sheep in 1984 (Chappuis *et al.*, 1986).
95 A second such isolate was a BDV contaminant of a live CSFV vaccine (Wensvoort & Terpstra, 1988).

96 **2. Fetal infection**

97 The main clinical signs of BD are seen following the infection of pregnant ewes. While the initial maternal
98 infection is subclinical or mild, the consequences for the fetus are serious. Fetal death may occur at any stage
99 of pregnancy, but is more common in fetuses infected early in gestation. Small dead fetuses may be resorbed
100 or their abortion may pass unnoticed as the ewes continue to feed well and show no sign of discomfort. As
101 lambing time approaches, the abortion of larger fetuses, stillbirths and the premature births of small, weak
102 lambs will be seen. Confirmation that an abortion or stillbirth is due to BDV is often difficult to establish, but
103 virus may be isolated from fetal tissues in some cases. The use of an appropriate real-time reverse-
104 transcription polymerase chain reaction (RT-PCR) assay may give a higher level of success because of the
105 advantages of high sensitivity and the ability to detect genome from non-infectious virus. In aborted fetuses, it
106 is also possible to detect virus by immunohistochemistry of brain, thyroid and other tissues (Thur *et al.*, 1997).
107 Samples of fetal fluids or serum should be tested for BDV antibody.

108 During lambing, an excessive number of barren ewes will become apparent, but it is the diseased live lambs
109 that present the main clinical features characteristic of BD. The clinical signs exhibited by BD lambs are very
110 variable and depend on the breed of sheep, the virulence of the virus and the time at which infection was
111 introduced into the flock. Affected lambs are usually small and weak, many being unable to stand. Nervous
112 signs and fleece changes are often apparent. The nervous signs of BD are its most characteristic feature. The
113 tremor can vary from violent rhythmic contractions of the muscles of the hindlegs and back, to barely detectable
114 fine trembling of the head, ears, and tail. Fleece abnormalities are most obvious in smooth-coated breeds,
115 which develop hairy fleeces, especially on the neck and back. Abnormal brown or black pigmentation of the
116 fleece may also be seen in BD-affected lambs. Blood samples to be tested for the presence of BDV or antibody
117 should be collected into anticoagulant from suspect lambs before they have received colostrum. Once lambs
118 have ingested colostrum, it is difficult to isolate virus until they are 2 months old and maternal antibody levels
119 have waned. However, during this period, it may be possible to detect viral antigen in skin biopsies, by
120 immunohistochemistry, in washed leukocytes by enzyme-linked immunosorbent assay (ELISA) or by real-time
121 RT-PCR. ELISAs directed at detection of the Erns antigen appear to be less prone to interference by maternal
122 antibodies and can often be used to detect antigen in serum.

123 With careful nursing, a proportion of BD lambs can be reared, although deaths may occur at any age. The
124 nervous signs gradually decline and can disappear by 3–6 months of age. Weakness, and swaying of the hind-
125 quarters, together with fine trembling of the head, may reappear at times of stress. Affected lambs often grow
126 slowly and under normal field conditions many will die before or around weaning time. In cases where losses
127 at lambing time have been low and no lambs with obvious signs of BD have been born, this can be the first
128 presenting sign of disease.

129 Some fetal infections occurring around mid-gestation can result in lambs with severe nervous signs, locomotor
130 disturbances and abnormal skeletons. Such lambs have lesions of cerebellar hypoplasia and dysplasia,
131 hydranencephaly and porencephaly resulting from necrotising inflammation. The severe destructive lesions
132 appear to be immune mediated, and lambs with such lesions frequently have high titres of serum antibody to
133 BDV. Most lambs infected in late gestation are normal and healthy and are born free from virus but with BDV
134 antibody. Some such lambs can be weak and may die in early life (Barlow & Patterson, 1982).

135 **3. Persistent viraemia**

136 When fetuses survive an infection that occurs before the onset of immune competence, they are born with a
137 persistent viraemia. The ovine fetus can first respond to an antigenic stimulus between approximately 60 and
138 85 days of its 150-day gestation period. In fetuses infected before the onset of immune competence, viral
139 replication is uncontrolled and 50% fetal death is common. In lambs surviving infection in early gestation, virus
140 is widespread in all organs. Such lambs appear to be tolerant of the virus and have a persistent infection,
141 usually for life. A precolostral blood sample will be virus positive and antibody negative. Typically, there is no
142 inflammatory reaction and the most characteristic pathological changes are in the central nervous system

143 (CNS) and skin. Throughout the CNS, there is a deficiency of myelin, and this causes the nervous signs. In
 144 the skin, primary wool follicles increase in size and the number of secondary wool follicles decreases, causing
 145 the hairy or coarse fleece.

146 Persistently viraemic sheep can be identified by the detection of viral antigens, nucleic acids or infectious virus
 147 in a blood sample. Viraemia is readily detectable by testing of serum at any time except within the first 2 months
 148 of life, when virus may be masked by colostral antibody and, possibly, in animals older than 4 years, some of
 149 which develop low levels of anti-BDV antibody (Nettleton *et al.*, 1992). Methods other than virus isolation may
 150 be preferred to avoid interference from antibodies. When the presence of colostral antibodies is suspected,
 151 the virus may be detected in washed leukocytes and in skin by using sensitive ELISAs. Although virus detection
 152 in blood during an acute infection is difficult, persistent viraemia should be confirmed by retesting animals after
 153 an interval of at least 3 weeks. The use of real-time RT-PCR should be considered at all times and for any
 154 sample type due to its high analytical sensitivity and the lack of interference from antibodies in a sample.

155 Some viraemic sheep survive to sexual maturity and are used for breeding. Lambs born to these infected dams
 156 are always persistently viraemic. Persistently viraemic sheep are a continual source of infectious virus to other
 157 animals and their identification is a major factor in any control programme. Sheep being traded should be
 158 screened for the absence of BDV viraemia.

159 Usually persistently infected (PI) rams have poor quality, highly infective semen and reduced fertility. All rams
 160 used for breeding should be screened for persistent BDV infection on a blood sample. Semen samples can
 161 also be screened for virus, but virus isolation is much less satisfactory than from blood because of the toxicity
 162 of semen for cell cultures. Real-time RT-PCR for detection of pestivirus nucleic acid would usually overcome
 163 toxicity problems, and thus this assay should be useful for testing semen from rams.

164 4. Late-onset disease in persistently viraemic sheep

165 Some PI sheep housed apart from other animals spontaneously develop intractable diarrhoea, wasting,
 166 excessive ocular and nasal discharges, sometimes with respiratory distress. At necropsy such sheep have
 167 gross thickening of the distal ileum, caecum and colon resulting from focal hyperplastic enteropathy. Cytopathic
 168 BDV can be recovered from the gut of these lambs. With no obvious outside source of cytopathic virus, it is
 169 most likely that such virus originates from the lamb's own virus pool, similar to what occurs with BVDV. Other
 170 PI sheep in the group ~~do~~ may not develop the disease. This syndrome, which has been produced
 171 experimentally and recognised in occasional field outbreaks of BD, has several similarities with bovine mucosal
 172 disease (Nettleton *et al.*, 1992).

173 B. DIAGNOSTIC TECHNIQUES

174 **Table 1.** Test methods available for diagnosis of border disease 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
Identification of the agent ^(a)						
Virus isolation	+	++	++	+++	–	–
Antigen detection by ELISA	+	++	+++	+++	–	–
NA detection by real-time RT-PCR	+++	+++	+++	+++	+++	–

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
NA detection by ISH	–	–	–	+	–	–
Detection of immune response						
Antibody detection by ELISA	++	++	++	+	++	++
VN	+++	+++	++	+++	+++	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.

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

^(a)A combination of agent identification methods applied on the same clinical sample is recommended.

175
176
177
178
179

180 1. Identification of the agent

181 There is no designated WOAHP Reference Laboratory for BDV, but the reference laboratories for BVDV or
182 CSFV will be able to provide advice¹. One of the most sensitive proven methods for identifying BDV remains
183 virus isolation. However, a broadly reactive real-time RT-PCR assay (preferably pan-pestivirus reactive) will
184 usually provide higher analytical sensitivity than virus isolation, can be used to test samples that are difficult to
185 manage by virus isolation and can be performed in a few hours. Antigen-detection ELISA and
186 immunohistochemical techniques on tissue sections are also valuable methods for identifying BDV-infected
187 animals.

188 1.1. Virus isolation

189 It is essential that laboratories undertaking virus isolation have a guaranteed supply of pestivirus-
190 free susceptible cells and bovine serum, or equivalent, that contain no anti-pestivirus activity and no
191 contaminating virus. It is important that a laboratory quality assurance programme be in place.
192 Chapter 3.4.7 provides detailed methods for virus isolation in either culture tubes or microplates for
193 the isolation of pestiviruses from sheep or goat samples, including serum, whole blood, semen and
194 tissues. The principles and precautions outlined in that chapter for the selection of cell cultures,
195 medium components and reagents are equally relevant to this chapter. Provided proven pan-
196 pestivirus reactive reagents (e.g. monoclonal antibodies [MAbs], primers and probes for real-time
197 RT-PCR) are used for antigen or nucleic acid detection, the principal difference is the selection of
198 appropriate cell cultures.

199 BD virus can be isolated in a number of primary or secondary ovine cell cultures (e.g. kidney, testes,
200 lung). Ovine cell lines for BDV growth are rare. Semicontinuous cell lines derived from fetal lamb
201 muscle (FLM), whole embryo (Thabti *et al.*, 2002) or sheep choroid plexus can be useful, but different
202 lines vary considerably in their susceptibility to the virus. Ovine cells have been used successfully
203 for the isolation and growth of BD viruses and BVDV types 1 and 2 from sheep. In regions where
204 sheep may become infected with BVD viruses from cattle, a virus isolation system using both ovine
205 and bovine cells could be optimal. However, bovine cells have lower sensitivity for the primary

¹ Please consult the WOAHP Web site: <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>

206 isolation and growth of some BD viruses, so reliance on bovine cells alone is inadvisable. Details of
207 suitable bovine cell cultures are provided chapter 3.4.7. The precautions outlined in that chapter for
208 the establishment of cells and medium components that are free from contamination with either
209 pestiviruses or antibodies, and measures to ensure that the cells are susceptible to a wide range of
210 local field strains are equally relevant to systems for detection of BDV.

211 From live animals, serum is the most frequently used sample to be tested for the presence of
212 infectious virus. However, for difficult cases, the most sensitive way to confirm pestivirus viraemia is
213 to wash leukocytes repeatedly (at least three times) in culture medium before co-cultivating them
214 with susceptible cells in either cell culture tubes or microplates. After culture for 5–7 days, the cultures
215 should be frozen and thawed once and an aliquot of diluted culture fluid passaged onto further
216 susceptible cells grown in microplates or on chamber slides to allow antigen detection by
217 immunocytochemistry. Staining for noncytopathic pestiviruses will usually detect virus at the end of
218 the primary passage, but to detect slow-growing viruses in poorly permissive cells two passages are
219 desirable. It is recommended that the culture supernatant used as inoculum for the second passage
220 is diluted approximately 1/100 in new culture medium because some high tited field isolates will
221 replicate poorly if passaged undiluted (i.e. at high multiplicity of infection – moi).

222 Tissues should be collected from dead animals in virus transport medium. In the laboratory, the
223 tissues are ground to give a 10–20% (w/v) suspension, centrifuged to remove debris, and the
224 supernatant passed through 0.45 µm filters. Spleen, lung, thyroid, thymus, kidney, brain, lymph
225 nodes and gut lesions are the best organs for virus isolation.

226 Semen can be examined for the presence of BDV, but raw semen is strongly cytotoxic and must be
227 diluted, usually at least 1/10 in culture medium. As the major threat of BDV-infected semen is from
228 PI rams, blood is a more reliable clinical sample than semen for identifying such animals. There are
229 many variations in virus isolation procedures. All should be optimised for maximum sensitivity using
230 a standard reference virus preparation and, whenever possible, recent BDV field isolates. Most of
231 the limitations of virus isolation for the detection of BDV in serum or blood, tissues or semen can be
232 overcome by the use of a proven, sensitive pan-pestivirus reactive real-time RT-PCR. Some
233 laboratories screen samples by real-time RT-PCR and undertake virus isolation on positive samples
234 to collect BDV strains for future reference or research purposes.

235 For specific technical details of virus isolation procedures, including immunoperoxidase staining,
236 refer to chapter 3.4.7.

237 **1.2. Nucleic acid detection methods**

238 The complete genomic sequences of three BD viruses have been determined and compared with
239 those of other pestiviruses (Becher *et al.*, 1998; Ridpath & Bolin, 1997). Phylogenetic analysis shows
240 BD viruses to be more closely related to CSFV than to BVDV (Becher *et al.*, 2003; Van Rijn *et al.*,
241 1997; Vilcek & Nettleton, 2006; Vilcek *et al.*, 1997). Real-time RT-PCR for diagnosing pestivirus
242 infection is now used widely and a number of formats have been described. Real-time RT-PCR
243 assays have the advantages of being able to detect both infectious virus and residual nucleic acid,
244 the latter being of value for investigating abortions and lamb deaths. Furthermore, the presence of
245 virus-specific antibodies in a sample will have no adverse effect on the sensitivity of the real-time
246 RT-PCR assay. These assays are also useful for screening semen and, when recommended nucleic
247 acid extraction protocols are followed, are less affected by components of the semen compared with
248 virus isolation. Because of the potential for small ruminants to be infected with genetically different
249 strains of BDV or with strains of BVDV, a ~~proven~~ pan-pestivirus reactive real-time RT-PCR with
250 proven high sensitivity should be used. To ensure that the genetic spectrum of BDV strains is
251 sufficiently covered, it may be necessary to apply a broadly reactive BDV specific real time RT-PCR
252 in parallel to maximise diagnostic sensitivity. Suitable protocols for both nucleic acid extraction as
253 well as the real-time RT-PCR are described in chapter 3.4.7. All precautions to minimise laboratory
254 contamination should be followed closely.

255 After testing samples in a pan-pestivirus reactive assay, samples giving positive results can any level
256 of reactivity should be investigated further by the application of a BDV-specific real-time RT-PCR
257 (Willoughby *et al.*, 2006). It is important to note however that different genotypes of BDV may be

258 circulating in some populations, especially wild ruminants such as chamois and deer, and may be
259 transferred to sheep. An assay that is specific for the detection of BDV should be used with some
260 caution as variants or previously unrecognised genotypes may not be detected, hence the value of
261 initially screening samples with a pan-pestivirus reactive real-time RT-PCR. Nevertheless, there are
262 also situations where a pan-pestivirus reactive real-time RT-PCR may have lower analytical
263 sensitivity. Consequently, in any situation where BDV infection is suspected, the application of
264 several diagnostic methods is recommended. Maternal serology can also play an important role as
265 negative results should exclude the potential involvement of a pestivirus.

266 **1.3. Enzyme-linked immunosorbent assay for antigen detection**

267 ELISAs for the direct detection of pestivirus antigen in blood and tissues of infected animals have
268 proven to be extremely useful for the detection of PI animals and the diagnosis of disease. The first
269 ELISA for pestivirus antigen detection was described for detecting viraemic sheep and was later
270 modified into a double MAb capture ELISA for use in sheep and cattle (Entrican *et al.*, 1994). The
271 test is most commonly employed to identify PI viraemic sheep using washed, detergent-lysed blood
272 leukocytes. The sensitivity is close to that of virus isolation and it is a practical method for screening
273 large numbers of blood samples. As with virus isolation, high levels of colostral antibody can mask
274 persistent viraemia. The ELISA is more effective than virus isolation in the presence of antibody, but
275 may give false-negative results in viraemic lambs younger than 2 months old. The ELISA is usually
276 not sensitive enough to detect acute BDV infections on blood samples. As well as for testing
277 leukocytes, the antigen ELISA can also be used on tissue suspensions, especially spleen, from
278 suspected PI sheep and, as an alternative to immunofluorescence and immunoperoxidase methods,
279 on cell cultures. Several pestivirus ELISA methods have been published but there are at present no
280 commercially available kits that have been fully validated for detecting BDV. Prior to use for
281 regulatory purposes, these kits should be validated in the region where they are to be used to ensure
282 that a wide range of field strains of BDV can be detected and that they are suitable for the sample
283 types to be tested.

284 **1.4. Immunohistochemistry**

285 Viral antigen demonstration is possible in most of the tissues of PI animals (Braun *et al.*, 2002; Thur
286 *et al.*, 1997) although this is not a method that is routinely used for diagnostic purposes. This should
287 be done on acetone-fixed frozen tissue sections (cryostat sections) or paraffin wax embedded
288 samples using appropriate antibodies. Pan-pestivirus reactive antibodies with NS2-3 specificity are
289 suitable. Tissues with a high amount of viral antigen are brain, thyroid gland, lung and oral mucosa.
290 Skin biopsies have been shown to be useful for *in-vivo* diagnosis of persistent BDV infection.

291 **2. Serological tests**

292 Antibody to BDV is usually detected in sheep sera using VN or an ELISA. The less sensitive agar gel
293 immunodiffusion test is not recommended. Control positive and negative reference sera must be included in
294 every test. These should give results within predetermined limits for the test to be considered valid. Single sera
295 can be tested to determine the prevalence of BDV in a flock, region or country. For diagnosis, however, acute
296 and convalescent sera are the best samples for confirming acute BDV infection. Repeat sera from one animal
297 should always be tested alongside each other on the same plate to provide a reliable comparison of titres.

298 **2.1. Virus neutralisation test**

299 Due to antigenic diversity among pestiviruses the choice of test virus is difficult (Dekker *et al.*, 1995;
300 Nettleton *et al.*, 1998). No single strain of BDV is ideal. A local strain that gives the highest antibody
301 titre with a range of positive sheep sera should be used.

302 Because there are few cytopathogenic strains of BDV available, to achieve optimal analytical
303 sensitivity, it is more usual to employ a representative local non-cytopathogenic strain and read the
304 assay after immunoperoxidase staining of the cells. Proven highly sensitive, pestivirus-free sheep
305 cells such as lamb testis or kidney cells are suitable and can be maintained as cryogenically frozen
306 stocks for use over long periods of time. The precautions outlined for selection of pestivirus-free

307 medium components are equally applicable to reagents to be used in VN tests. A recommended
308 procedure follows.

309 **2.1.1. Test procedure**

- 310 i) The test sera are heat-inactivated for 30 minutes at 56°C.
- 311 ii) From a starting dilution of 1/4, serial twofold dilutions of the test sera are made in a cell-
312 culture grade flat-bottomed 96-well microtitre plate, using cell culture medium as diluent.
313 For each sample, three or four wells are used at each dilution depending on the degree
314 of precision required. Also, for each sample and at each serum dilution, one well is left
315 without virus to monitor for evidence of sample toxicity that could mimic viral
316 cytopathology or interfere with virus replication. Control positive and negative sera should
317 also be included in each batch of tests.
- 318 iii) An equal volume (e.g. 50 µl) of a stock of BDV containing 100 TCID₅₀ (50% tissue culture
319 infective dose) is added to each well. A back titration of virus stock is also done in some
320 spare wells to check the potency of the virus (acceptance limits **30-80**–300 TCID₅₀).
- 321 iv) The plate is incubated for 1 hour at 37°C.
- 322 v) A flask of suitable cells (e.g. ovine testis or kidney cells) is trypsinised and the cell
323 concentration is adjusted to 2 × 10⁵/ml. 100 µl of the cell suspension is added to each
324 well of the microtitre plate.
- 325 vi) The plate is incubated at 37°C for 4–5 days, either in a 5% CO₂ atmosphere or with the
326 plate sealed.
- 327 vii) The wells are examined microscopically to ensure that there is no evidence of toxicity or
328 cytopathic effect (CPE), then fixed and stained by immunoperoxidase staining using an
329 appropriate MAb. The VN titre for each serum is the dilution at which the virus is
330 neutralised in 50% of the wells. This can be calculated by the Spearman–Kärber or Reed
331 Muench methods. A seronegative animal will show no neutralisation at the lowest dilution
332 of serum (i.e. 1/4), equivalent to a final dilution of 1/8. For accurate comparison of
333 antibody titres, and particularly to demonstrate significant (more than fourfold) changes
334 in titre, samples should be tested in parallel in the same test.
- 335 viii) Occasionally there may be a need to determine whether antibody in a flock is against a
336 virus belonging to a particular *Pestivirus* serogroup. A differential VN test can be used in
337 which sera are titrated out against representative viruses from each of the four *Pestivirus*
338 groups, i.e. BDV, BVDV types 1 and 2, and CSFV. Maximum titre will identify the infecting
339 serotype and the spectrum of cross-reactivity with the other serotypes will also be
340 revealed.

341 **2.2. Enzyme-linked immunosorbent assay**

342 An MAb-capture ELISA for measuring BDV antibodies has been described. Two pan-pestivirus MAbs
343 that detect different epitopes on the immunodominant nonstructural protein NS 2/3 are used to
344 capture detergent-lysed cell-culture grown antigen. The results correlate qualitatively with the VN
345 test (Fenton *et al.*, 1991).

346 **2.2.1. Antigen preparation**

347 Use eight 225 cm² flasks of newly confluent FLM cells; four flasks will be controls and four will
348 be infected. Wash the flasks and infect four with a 0.01–0.1 m.o.i. of Moredun cytopathic BDV.
349 Allow the virus to adsorb for 2 hours at 37°C. Add maintenance media containing 2% FBS
350 (free from BDV antibody), and incubate cultures for 4–5 days until CPE is obvious. Pool four
351 control flask supernatants and separately pool four infected flask supernatants. Centrifuge at
352 3000 **g** for 15 minutes to pellet cells. Discard the supernatants. Retain the cell pellets. Wash
353 the flasks with 50 ml of PBS and repeat the centrifugation step as above. Pool all the control
354 cell pellets in 8 ml PBS containing 1% Nonidet P40 and return 2 ml to each control flask to
355 lyse the remaining attached cells. Repeat for infected cells. Keep the flasks at 4°C for at least
356 2 hours agitating the small volume of fluid on the cells vigorously every 30 minutes to ensure

357 total cell detachment. Centrifuge the control and infected antigen at 12,000 **g** for 5 minutes to
358 remove the cell debris. Supernatant antigens are stored at –70°C in small aliquots.

359 **2.2.2. Test procedure**

- 360 i) The two MAbs are diluted to a predetermined dilution in 0.05 M bicarbonate buffer, pH
361 9.6. All wells of a suitable ELISA-grade microtitre plate (e.g. Nunc maxisorb, Greiner
362 129b) are coated overnight at 4°C.
- 363 ii) After washing three times in PBST, a blocking solution of PBST containing 10% horse
364 serum (PBSTH) is added to all wells, which are incubated at 37°C for 1 hour.
- 365 iii) The antigen is diluted to a predetermined dilution in PBSTH and alternate rows of wells
366 are coated with virus and control antigens for 1 hour at 37°C. The plates are then washed
367 three times in PBST before addition of test sera.
- 368 iv) Test sera are diluted 1/50 in PBSTH and added to duplicate virus and duplicate control
369 wells for 1 hour at 37°C. The plates are then washed three times in PBST.
- 370 v) Anti-ovine IgG peroxidase conjugate is diluted to a predetermined dilution in PBSTH and
371 added to all wells for 1 hour at 37°C. The plates are washed three times in PBST.
- 372 vi) A suitable activated enzyme substrate/chromogen, such as ortho-phenylene diamine
373 (OPD) or tetramethyl benzidine (TMB), is added). After colour development, the reaction
374 is stopped with sulphuric acid and the absorbance read on an ELISA plate reader. The
375 mean value of the two control wells is subtracted from the mean value of the two virus
376 wells to give the corrected absorbance for each serum. Results are expressed as
377 corrected absorbance with reference to the corrected absorbance of known positive and
378 negative sera. Alternatively, ELISA titres can be extrapolated from a standard curve of a
379 dilution series of a known positive reference serum.
- 380 If antigens of sufficient potency can be produced the MAb capture stage can be omitted.
381 In this case alternate rows of wells are coated with virus and control antigen diluted to a
382 predetermined dilution in 0.05 M bicarbonate buffer, pH 9.6, overnight at +4°C. The plates
383 are washed and blocked as in step ii above. After washing, diluted test sera are added
384 and the test proceeds from step iv as above.

385 **C. REQUIREMENTS FOR VACCINES**

386 **1. Background**

387 To be useful, a BDV vaccine should be effective when administered to female sheep before breeding to prevent
388 transplacental infection. Experimental and commercial inactivated whole virus BDV vaccines have been
389 produced in Europe (Brun *et al.*, 1993; Vantsis *et al.*, 1980). Unlike vaccines for BVDV, there is limited demand
390 for vaccines against BDV and those produced have only been inactivated products. No live attenuated or
391 recombinant subunit vaccines for BDV have been produced commercially.

392 Pestivirus contaminants of modified live virus vaccines have been found to be a cause of serious disease
393 following their use in pigs, cattle, sheep and goats. Contaminated vaccines have included those used for the
394 control of Aujeszky's disease, CSF, rotavirus, coronavirus, rinderpest, sheep pox and contagious pustular
395 dermatitis. The insidious ability of pestiviruses to cross the placenta, and thus establish PI animals, gives them
396 the potential to contaminate vaccines through cells, serum used as medium supplement, or seed stock virus.
397 As nearly all isolates of pestiviruses are noncytopathic, they will remain undetected unless specific tests are
398 carried out. Although such contamination should be less likely to be a problem with an inactivated vaccine,
399 nevertheless steps should be taken to ensure that materials used in production are not contaminated.

400 **1.1. Characteristics of a target product profile**

401 Traditionally, pestivirus vaccines fall into two classes: modified live or inactivated virus vaccines. The
402 essential requirement for both types is to **afford-provide** a high level of fetal infection. Only inactivated
403 vaccines have been produced for BDV. Properly formulated inactivated vaccines are very safe to
404 use but, to obtain satisfactory levels of immunity, they usually require booster vaccinations, which
405 may be inconvenient. Because of the propensity for antigenic variability, the vaccine should contain
406 strains of BDV that are closely matched to viruses found in the area in which they are used. This
407 may present particular challenges with BDV in regions where several antigenic types have been
408 found. Due to the need to customise vaccines for the most commonly encountered strains within a
409 country or region, it is not feasible to produce a vaccine antigen bank that can be drawn upon globally.

410 Guidance for the production of veterinary vaccines is given in Chapter 1.1.8 *Principles of veterinary*
411 *vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in
412 nature and may be supplemented by national and regional requirements.

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

414 **2.1. Characteristics of the seed**

415 An ideal vaccine should contain a strain or strains of virus that give protection against all sheep
416 pestiviruses. This may be challenging however, because of the range of pestiviruses with which
417 sheep can be infected. There is considerable antigenic variation across these viruses – both between
418 viruses that have been classified in the BDV genogroup as well as between viruses in the BVDV1
419 and BVDV2 genotypes (Becher *et al.*, 2003; Vilcek & Nettleton, 2006; Wensvoort *et al.*, 1989).
420 Infection of sheep with the putative BVDV-3 genotype has also been described (Decaro *et al.*, 2012).
421 It is likely that the antigenic composition of a vaccine will vary from region to region to provide an
422 adequate antigenic match with dominant virus strains. Cross-neutralisation studies are required to
423 establish optimal combinations. Nevertheless, it would appear that any BDV vaccine should contain
424 at least a representative of the BDV and BVDV (type 1) groups. Characterisation of the biologically
425 cloned vaccine viruses should include typing with MAbs and genotyping (Paton *et al.*, 1995).

426 **2.1.1. Quality criteria (sterility, purity, freedom from extraneous agents)**

427 It is crucial to ensure that all materials used in the preparation of the bulk antigens have been
428 extensively screened to ensure freedom from extraneous agents. This should include master
429 and working seeds, the cell cultures and all medium supplements such as bovine serum. Some
430 bovine viruses and particularly BVDV can readily infect small ruminants such as sheep.
431 Therefore, it is particularly important to ensure that any serum used that is of bovine origin is
432 free of both adventitious BVDV and antibodies against BVDV strains because low levels of
433 either virus or antibody can mask the presence of the other. Materials and vaccine seeds
434 should be tested for sterility and freedom from contamination with other agents, especially
435 viruses as described in the chapter 1.1.8 and Chapter 1.1.9 *Tests for sterility and freedom*
436 *from contamination of biological materials intended for veterinary use*.

437 If a vaccine passes basic tests, the efficacy of vaccination should ultimately be measured by
438 the capacity to prevent transplacental transmission. Effective challenge of vaccinated
439 pregnant ewes at 50–60 days gestation has been achieved by intranasal installation of virus
440 or by mixing with PI sheep (Brun *et al.*, 1993). Usually this reliably produces persistently
441 viraemic offspring in non-immune ewes. In regions where multiple genotypes of BDV viruses
442 are commonly encountered, efficacy in protecting against multiple strains should be measured.

443 **2.2. Method of manufacture**

444 **2.2.1. Procedure**

445 Inactivated vaccines have been prepared using conventional laboratory techniques with
446 stationary or rolled cell cultures. Inactivants have included formalin and beta-propiolactone.
447 Adjuvants have included aluminium hydroxide and oil (Brun *et al.*, 1993; Vantsis *et al.*, 1980).
448 Optimal yields depend on the cell type and isolate used. A commercial BDV vaccine containing

449 two strains of virus has been prepared on ovine cell lines (Brun *et al.*, 1993). Cells must be
450 produced according to a seed-lot system from a master cell seed (MCS) that has been shown
451 to be free from all contaminating microorganisms. Vaccine should only be produced in cells
452 fewer than 20 passages from the MCS. Control cells from every passage should be checked
453 for pestivirus contamination. Standard procedures may be used, with the expectation for
454 harvesting noncytopathic virus on days 4–7 after inoculation of cultures. The optimal yield of
455 infectious virus will depend on several factors, including the cell culture, isolate used and the
456 initial seeding rate of virus. These factors should be taken into consideration and virus
457 replication kinetics investigated to establish the optimal conditions for large-scale virus
458 production. Whether a live or inactivated vaccine, the essential aim will be to produce a high-
459 titred virus stock. This bulk antigen preparation can subsequently be prepared according to
460 the type of vaccine being considered.

461 **2.2.2. Requirements for ingredients**

462 BDV vaccines have usually been grown in cell cultures of ovine origin that are frequently
463 supplemented with medium components of animal origin. The material of greatest concern is
464 bovine serum due to the potential for contamination with BVD viruses and antibodies to these
465 viruses. These adventitious contaminants not only affect the efficiency of production but also
466 may mask the presence of low levels of infectious BVDV that may have undesirable
467 characteristics. In addition to the virus seeds, all materials should be tested for sterility and
468 freedom from contamination with other agents, especially viruses as described in chapters
469 1.1.8 and 1.1.9. Furthermore, materials of bovine or ovine origin should originate from a
470 country with negligible risk for transmissible spongiform encephalopathies (see chapter 1.1.9).

471 **2.2.3. In-process controls**

472 In-process controls are part of the manufacturing process. Cultures should be inspected
473 regularly to ensure that they remain free from gross bacterial contamination, and to monitor
474 the health of the cells and the development or absence of CPE, as appropriate. While the
475 basic requirement for efficacy is the capacity to induce an acceptable neutralising antibody
476 response, during production, target concentrations of antigen required to achieve an
477 acceptable response may be monitored indirectly by assessment of the quantity of infectious
478 virus or antigen mass that is produced. Rapid diagnostic assays such as the ELISA are useful
479 for monitoring BVDV antigen production. Alternatively, the quality of a batch of antigen may
480 be determined by titration of the quantity of infectious virus present, although this may
481 underestimate the quantity of antigen. For inactivated vaccines, infectivity is evaluated before
482 inactivation. For inactivated vaccines the inactivation kinetics should be established so that a
483 suitable safety margin can be determined and incorporated into the routine production
484 processes. At the end of production, *in-vitro* cell culture assays should be undertaken to
485 confirm that inactivation has been complete. These innocuity tests should include a sufficient
486 number of passages and volume of inoculum to ensure that very low levels of infectious virus
487 would be detected if present.

488 **2.2.4. Final product batch tests**

489 i) Sterility

490 Tests for sterility and freedom from contamination of biological materials intended for
491 veterinary use may be found in chapter 1.1.9.

492 ii) Identity

493 Identity tests should demonstrate that no other strain of BDV is present when several
494 strains are propagated in a facility producing multivalent vaccines.

495 iii) Safety

496 Samples from inactivated vaccines should be tested rigorously for viable virus. Samples
497 of the product should be passaged for a minimum of three passages in sensitive cell
498 cultures to ensure absence of live BDV. This *in-vitro* monitoring can be augmented by
499 injecting two BDV-seronegative sheep with 20 doses of unformulated antigen as part of

500 a standard safety test. Presence of live virus will result in the development of a more
501 convincing serological response than will occur with inactivated virus alone. The sheep
502 sera can also be examined for antibody to other prescribed agents.

503 Safety tests shall also consist of detecting any abnormal local or systemic adverse
504 reactions to the vaccine by all vaccination route(s). Batch-to-batch safety tests are
505 required unless safety of the product is demonstrated and approved in the registration
506 dossier and production is consistent with that described in chapter 1.1.8. Vaccines must
507 either be demonstrated to be safe in pregnant sheep (i.e. no transmission to the fetus),
508 or should be licensed with a warning not to use them in pregnant animals.

509 iv) Batch potency

510 Vaccine potency is best tested in seronegative sheep in which the development and level
511 of antibody is measured. BVD vaccines must be demonstrated to produce adequate
512 immune responses when used in their final formulation according to the manufacturer's
513 published instructions. The minimum quantity of infectious virus or antigen required to
514 produce an acceptable immune response should be determined. An indirect measure of
515 potency is given by the level of virus infectivity prior to inactivation. *In-vitro* assays should
516 be used to monitor individual batches during production. The antigen content following
517 inactivation can be assayed by MAb-capture ELISA and related to the results of
518 established *in-vivo* potency results. It should be demonstrated that the lowest
519 recommended dose of vaccine can prevent transplacental transmission of BDV in
520 pregnant sheep.

521 **2.3. Requirements for authorisation/registration/licensing**

522 **2.3.1. Manufacturing process**

523 For registration of a vaccine, all relevant details concerning manufacture of the vaccine and
524 quality control testing should be submitted to the relevant authorities. Unless otherwise
525 specified by the authorities, information should be provided from three consecutive vaccine
526 batches with a volume not less than 1/3 of the typical industrial batch volume.

527 There is no standard method for the manufacture of a BDV vaccine, but conventional
528 laboratory techniques with stationary, rolled or suspension (micro-carriers) cell cultures may
529 be used. Inactivated vaccines can be prepared by conventional methods, such as binary
530 ethylenimine, formalin or beta-propiolactone inactivation (Park & Bolin, 1987). A variety of
531 adjuvants may be used.

532 **2.3.2. Safety requirements**

533 *In-vivo* tests should be undertaken using repeat doses (taking into account the maximum
534 number of doses for primary vaccination and, if appropriate, the first revaccination/booster
535 vaccination) and contain the maximum permitted antigen load and, depending on the
536 formulation of the vaccine, the maximum number of vaccine strains.

537 i) Target and non-target animal safety

538 The safety of the final product formulation of inactivated vaccines should be assessed in
539 susceptible young sheep that are free of maternally derived antibodies and in pregnant
540 ewes. They should be checked for any local reactions following administration, and, in
541 pregnant ewes, for any effects on the unborn lamb.

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

543 In the event that a live virus vaccine was developed for BDV, virus seeds that have been
544 passaged at least up to and preferably beyond the passage limit specified for the seed
545 should be inoculated into young lambs to confirm that there is no evidence of disease. If
546 a live attenuated vaccine has been registered for use in pregnant animals, reversion to

547 virulence tests should also include pregnant animals. Live attenuated vaccines should
548 not be transmissible to unvaccinated 'in-contact' animals.

549 iii) Precautions (hazards)

550 BDV is not considered to be a human health hazard. Standard good microbiological
551 practice should be adequate for handling the virus in the laboratory. While the inactivated
552 virus in a vaccine should be identified as harmless for people administering the product,
553 adjuvants included in the vaccine may cause injury to people. Manufacturers should
554 provide adequate warnings that medical advice should be sought in the case of self-
555 injection (including for adjuvants, oil-emulsion vaccine, preservatives, etc.) with warnings
556 included on the product label/leaflet so that the vaccinator is aware of any danger.

557 **2.3.3. Efficacy requirements**

558 The potency of the vaccine should be determined by inoculation into seronegative and virus
559 negative lambs, followed by monitoring of the antibody response. Antigen content can be
560 assayed by infectivity titration prior to inactivation and subsequently by ELISA and adjusted
561 as required to a standard level for the particular vaccine. Standardised assay protocols
562 applicable to all vaccines do not exist. Live vaccine batches may be assayed by infectivity
563 titration. Each production batch of vaccine should undergo potency and safety testing as batch
564 release criteria. BVD vaccines must be demonstrated to produce adequate immune
565 responses, as outlined above, when used in their final formulation according to the
566 manufacturer's published instructions.

567 **2.3.4. Vaccines permitting a DIVA strategy (detection of infection in vaccinated animals)**

568 To date, there are no commercially available vaccines for BDV that support use of a true DIVA
569 strategy.

570 **2.3.5. Duration of immunity**

571 Inactivated vaccines are unlikely to provide sustained levels of immunity and it is likely that after an
572 initial course of two or three injections annual booster doses may be required. Insufficient information
573 is available to determine any correlation between vaccinal antibody titres in the dam and fetal
574 protection. As there are likely to be different commercial formulations and these involve a range of
575 adjuvants, there are likely to be different periods of efficacy. Consequently, duration of immunity data
576 must be generated separately for each commercially available product by undertaking challenge
577 tests at the end of the period for which immunity has been claimed.

578 **2.3.6. Stability**

579 There are no accepted guidelines for the stability of BDV vaccines, but it can be assumed that an
580 inactivated virus vaccine should remain potent for at least 1 year if kept at 4°C and probably longer.
581 Lower temperatures could prolong shelf life but adjuvants in a killed vaccine may preclude this. Bulk
582 antigens that have not been formulated into finished vaccine can be reliably stored frozen at low
583 temperatures, but the antigen quality should be monitored with *in-vitro* assays prior to incorporation
584 into a batch of vaccine.

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659 *
660 * *

661 **NB:** At the time of publication (2017) there were no WOA Reference Laboratories
662 for border disease (please consult the WOA Web site:
663 <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3http://www.oie.int/>).

664 **NB:** FIRST ADOPTED IN 1996. MOST RECENT UPDATES ADOPTED IN 2017.

Annexe 15. Chapter 3.8.12. 'Sheep pox and goat pox'

CHAPTER 3.8.12.

SHEEP POX AND GOAT POX

SUMMARY

Sheep pox and goat pox are contagious, viral diseases of sheep and goats characterised by fever, generalised papules or nodules, vesicles (rarely), internal lesions (particularly in the lungs), and death. Both diseases are caused by strains of capripoxvirus, all of which can infect sheep and goats. Although most of the strains examined cause more severe clinical disease in either sheep or goats, some strains have been isolated that are equally pathogenic in both species.

Sheeppox virus (SPPV) and goatpox virus (GTPV) are the causative agents of sheep pox and goat pox, and with lumpy skin disease virus (LSDV) make up the genus Capripoxvirus in the family Poxviridae. Sheep pox and goat pox are endemic in Africa north of the Equator, the Middle East and Asia, while some parts of Europe have experienced outbreaks recently. See WAHIS (<https://wahis.woah.org/#/home>) for recent information on distribution at the country level. ~~Countries that reported outbreaks of the disease between 2010 and 2015 include Bulgaria, Chinese Taipei, Israel, Kazakhstan, Kyrgyzstan, Mongolia, Morocco, Greece and Russia, with Greece, Israel and Russia having experienced recurring incidences.~~

Identification of the agent: Laboratory confirmation of capripoxvirus is most rapid using the polymerase chain reaction (PCR) method in combination with a clinical history consistent with generalised capripoxvirus infection. Isolation of the virus is possible as capripoxviruses will grow on tissue culture of ovine, caprine or bovine origin, although field isolates may require up to 14 days to grow or require one or more additional tissue culture passage(s). The virus causes intracytoplasmic inclusions that can be clearly seen using haematoxylin and eosin staining. The antigen can also be detected in tissue culture using specific sera and immunoperoxidase or immunofluorescence techniques. Capripoxvirus antigen and inclusion bodies may be seen in stained cryostat or paraffin sections of biopsy or post-mortem lesion material.

~~An antigen detection enzyme-linked immunosorbent assay (ELISA) using a polyclonal detection serum raised against a recombinant immunodominant antigen of capripoxvirus has been developed.~~

Serological tests: The virus neutralisation test is the most specific serological test. The indirect immunofluorescence test is less specific due to cross-reactions with antibody to other poxviruses. Western blotting using the reaction between the P32 antigen of capripoxvirus with test sera is both sensitive and specific, but is expensive and difficult to carry out. An enzyme-linked immunosorbent assay (ELISA) has been developed and validated to detect antibodies to capripoxviruses, however it cannot differentiate between SPPV, GTPV and LSDV.

~~The use of this antigen, or other appropriate antigens expressed by a suitable vector, in an ELISA offers the prospect of an acceptable and standardised serological test in the future.~~

37 **Requirements for vaccines:** Live and inactivated vaccines have been used for the control of
38 capripoxviruses. All strains of capripoxvirus so far examined share a major neutralisation site and
39 some will cross protect. Inactivated vaccines give, at best, only short-term immunity.

40 A. INTRODUCTION

41 The *Capripoxvirus* genus, in the family *Poxviridae*, consists of three species – lumpy skin disease virus (LSDV),
42 which causes disease in cattle only (see Chapter 3.4.12), and sheeppox virus (SPPV) and goatpox virus
43 (GTPV), which cause sheep pox and goat pox, respectively. Sheep pox and goat pox are characterised by
44 disseminated cutaneous nodules and up to 100% mortality in fully susceptible breeds naïve of sheep and
45 goats. In indigenous animals, generalised disease and mortality are less common, although they are seen
46 where disease has been absent from an area or village for a period of time, when intensive husbandry methods
47 are introduced, or in association with other disease agents, such as peste des petits ruminants virus or foot
48 and mouth disease virus. Sheep pox and goat pox are major constraints to the introduction of exotic breeds of
49 sheep and goats to endemic areas, and to the development of intensive livestock production.

50 Strains of SPPV and GTPV can pass between sheep and goats, although most cause more severe clinical
51 disease in ~~only one~~ their homologous host species. SPPV and GTPV are transboundary diseases that regularly
52 spread into adjacent, non-endemic areas. Sheep pox and goat pox are endemic in Africa north of the Equator
53 and parts of the Middle East and Asia (see WAHIS for most up-to-date information on distribution:
54 <https://wahis.woah.org/#/home>). Outbreaks have been reported in non-endemic countries of Asia, Europe and
55 the Middle East.

56 The incubation period of sheep pox and goat pox is between 8 and 13 days following contact between infected
57 and susceptible animals. It may be as short as 4 days following experimental infection by intradermal
58 inoculation ~~or mechanical transmission by insects~~. Some breeds of European sheep, such as Soay, may die
59 of acute infection before the development of skin lesions. In other breeds there is an initial rise in rectal
60 temperature to above 40°C, followed in 2–5 days by the development of, at first, macules – small circumscribed
61 areas of hyperaemia, which are most obvious on unpigmented skin – and then of papules – hard swellings of
62 between 0.5 and 1 cm in diameter – which may cover the body or be restricted to the groin, axilla and perineum.
63 Papules may be covered by fluid-filled vesicles, but this is rare. Some researchers have distinguished between
64 a vesicular and nodular form of sheep pox and goat pox (Zro *et al.*, 2014b).

65 Within 24 hours of the appearance of generalised papules, affected animals develop rhinitis, conjunctivitis and
66 enlargement of all the superficial lymph nodes, in particular the prescapular lymph nodes. Papules on the
67 eyelids cause blepharitis of varying severity. As the papules on the mucous membranes of the eyes and nose
68 ulcerate, so the discharge becomes mucopurulent, and the mucosae of the mouth, anus, and prepuce or
69 vagina become necrotic. Breathing may become laboured and noisy due to pressure on the upper respiratory
70 tract from the swollen retropharyngeal lymph nodes, due to the developing lung lesions.

71 If the affected animal does not die in this acute phase of the disease, the papules start to become necrotic
72 from ischaemic necrosis following thrombi formation in the blood vessels at the base of the papule. In the
73 following 5–10 days the papules form scabs, which persist for up to 6 weeks, leaving small scars. The skin
74 lesions are susceptible to fly strike, and secondary pneumonia is common. Anorexia is not usual unless the
75 mouth lesions physically interfere with feeding. Abortion is rare.

76 On post-mortem examination of the acutely infected animal, the skin lesions are often less obvious than on
77 the live animal. The mucous membranes appear necrotic and all the body lymph nodes are enlarged and
78 oedematous. Papules, which may be ulcerated, can usually be found on the abomasal mucosa, and
79 sometimes on the wall of the rumen and large intestine, on the tongue, hard and soft palate, trachea and
80 oesophagus. Pale areas of approximately 2 cm in diameter may occasionally be seen on the surface of the
81 kidney and liver, and have been reported to be present in the testicles. Numerous hard lesions of up to 5 cm
82 in diameter are commonly observed throughout the lungs, but particularly in the diaphragmatic lobes.

83 The clinical signs and post-mortem lesions vary considerably with breed of host and strain of capripoxvirus.
84 Indigenous breeds are less susceptible and frequently show only a few lesions, which could be confused with
85 insect bites or contagious pustular dermatitis. However, lambs that have lost their maternally derived immunity,

86 animals that have been kept isolated and animals brought into endemic areas from isolated villages,
 87 particularly if they have been subjected to the stress of moving long distances and mixing with other sheep
 88 and goats, and their pathogens, can often be seen with generalised and sometimes fatal capripoxvirus
 89 infections. Invariably there is high mortality in unprotected imported breeds of sheep and goats following
 90 capripoxvirus infection. Surviving animals clear the infection, as there is no evidence of persistently infected
 91 animals. Capripoxvirus is not infectious to humans. Capripoxvirus is inactivated at 56°C for 2 hours or 65°C
 92 for 30 minutes. The virus survives between pH 6.6–8.6. It is susceptible to highly alkaline or acid pH. The virus
 93 is sensitive to various chemicals: sodium dodecyl sulphate, ether 20%, chloroform, formalin 1%, sodium 2%,
 94 iodine compounds, Virkon 2%, quaternary ammonium (0.5%), and phenol 2% for 15 minutes.

95 B. DIAGNOSTIC TECHNIQUES

96 **Table 1. Test methods available for diagnosis of sheep pox and goat pox 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
Identification of the agent^(a)						
Virus isolation	+	++	+	+++	+	–
Antigen detection	++	++	++	++	++	–
<u>IFAT</u>	<u>±</u>	<u>±</u>	<u>±</u>	<u>++</u>	<u>±</u>	≡
<u>IHC</u>	<u>±</u>	<u>±</u>	<u>±</u>	<u>++</u>	<u>±</u>	≡
PCR	++	+++	++	+++	++	–
Detection of immune response						
<u>VNT</u>	++	++	++	++	++	++
IFAT	+	+	+	+	+	+
<u>ELISA</u>	<u>++</u>	<u>++</u>	<u>++</u>	<u>++</u>	<u>++</u>	<u>++</u>

97 Key: +++ = recommended for this purpose; ++ recommended but has limitations;

98 + = suitable in very limited circumstances; – = not appropriate for this purpose.

99 IFAT = indirect fluorescent antibody test; IHC = immunohistochemistry; PCR = polymerase chain reaction;

100 VNT = virus neutralisation; ELISA = enzyme-linked immunosorbent assay.

101 ^(a)A combination of agent identification methods applied on the same clinical sample is recommended.

102 1. Identification of the agent

103 1.1. Specimen collection and submission

104 Material for virus isolation and antigen detection should be collected by biopsy or at post-mortem
 105 from skin papules, lung lesions or lymph nodes. Samples for virus isolation and antigen detection
 106 enzyme-linked immunosorbent assay (ELISA) should be collected within the first week of the
 107 occurrence of clinical signs, before the development of neutralising antibodies. Samples for genome
 108 detection by polymerase chain reaction (PCR) may be collected before or after the development of
 109 neutralising antibody responses. In addition to epithelial lesions, nasal and buccal swabs can be

110 collected because the virus will be present in nasal and saliva discharges. Buffy coat from blood
111 collected into EDTA (ethylene diamine tetra-acetic acid) during the viraemic stage of capripoxvirus
112 infection (before generalisation of lesions or within 4 days of generalisation), can also be used for
113 virus isolation.

114 Samples for histology should include tissue from the surrounding area and should be placed
115 immediately following collection into ten times the sample volume of 10% formalin or neutral buffered
116 10% formal saline. Tissues in formalin have no special transportation requirements.

117 Blood samples, for virus isolation from the buffy coat, should be collected in tubes containing
118 anticoagulant, placed immediately on ice and processed as soon as possible. In practice, the blood
119 samples may be kept at 4°C for up to 2 days prior to processing, but should not be frozen or kept at
120 ambient temperatures. Tissues and dry scabs for virus isolation, antigen detection and genome
121 detection should preferably be kept at 4°C, on ice or at -20°C. If it is necessary to transport samples
122 over long distances without refrigeration, the medium should contain 10% glycerol; the samples
123 should be of sufficient size that the transport medium does not penetrate the central part of the
124 biopsy, which should be used for virus isolation/detection.

125 1.2. Virus isolation

126 Lesion material for virus isolation and genome antigen detection is homogenised. The following is an
127 example of one technique for homogenisation: The tissue is minced using sterile scissors and
128 forceps, and then macerated in a steel ball bearing mixer mill or ground with a sterile pestle in a
129 mortar with sterile sand and an equal volume of sterile phosphate buffered saline (PBS) or serum-
130 free Modified Eagle's Medium (MEM) containing sodium penicillin (1000 international units [IU]/ml),
131 streptomycin sulphate (1 mg/ml), mycostatin (100 IU/ml) or fungizone (2.5 µg/ml) and neomycin
132 (200 IU/ml). The homogenised suspension is freeze-thawed three times and then partially clarified
133 by centrifugation using a bench centrifuge at 600 **g** for 10 minutes. In cases where bacterial
134 contamination of the sample is expected (such as when virus is isolated from skin samples), the
135 supernatant can be filtered through a 0.45 µm pore size filter after the centrifugation step, however,
136 the amount of virus in the supernatant might be reduced. Buffy coats may be prepared from 5–8 ml
137 unclotted blood by centrifugation at 600 **g** for 15 minutes; the buffy coat is carefully removed into 5
138 ml of cold double-distilled water using a sterile Pasteur pipette. After 30 seconds, 5 ml of cold double-
139 strength growth medium is added and mixed. The mixture is centrifuged at 600 **g** for 15 minutes, the
140 supernatant is discarded and the cell pellet is suspended in 5 ml of growth medium, such as
141 Glasgow's modified Eagle's medium (GMEM). After centrifugation at 600 **g** for a further 15 minutes,
142 the resulting pellet is suspended in 5 ml of fresh GMEM. Alternatively, the buffy coat may be
143 separated from a heparinised sample using a density gradient.

144 Capripoxvirus will grow in tissue culture of bovine, ovine or caprine origin, although primary or
145 secondary cultures of lamb testis (LT) or lamb kidney (LK) cells are considered to be the most
146 susceptible. Care needs to be taken to ensure they are not contaminated with viruses such as bovine
147 viral diarrhoea virus, particularly those derived from a wool sheep breed (see chapter 1.1.9). Madin-
148 Darby bovine kidney (MDBK) cells have been shown to be suitable for capripoxvirus isolation (Fay
149 et al., 2020). The following is an example of an isolation technique: either 1 ml of buffy coat cell
150 suspension or 1 ml of clarified biopsy preparation supernatant is inoculated on to a 25 cm² tissue
151 culture flask of appropriate cells at 90% confluent LT or LK cells confluence, and the supernatant is
152 allowed to adsorb for 1 hour at 37°C. The culture is then washed with warm PBS and covered with
153 10 ml of a suitable medium, such as GMEM, containing antibiotics and 2% fetal calf serum. If
154 available, tissue culture tubes containing LT or LK cells and a, flying cover-slips, or tissue culture
155 microscope slides, are can also be infected.

156 The flasks should be examined daily for 7–14 days for evidence of cytopathic effect (CPE).
157 Contaminated flasks should be discarded. Infected cells develop a characteristic CPE consisting of
158 retraction of the cell membrane from surrounding cells, and eventually rounding of cells and
159 margination of the nuclear chromatin. At first only small areas of CPE can be seen, sometimes as
160 soon as 4 days after infection; over the following 4–6 days these expand to involve the whole cell
161 sheet. If no CPE is apparent by day 7, the culture should be freeze-thawed three times, and clarified
162 supernatant inoculated on to fresh LT or LK cell cultures. At the first sign of CPE in the flasks, or

163 earlier if a number of infected cover-slips are being used, a cover-slip should be removed, fixed in
164 acetone and stained using H&E. Eosinophilic intracytoplasmic inclusion bodies, which are variable
165 in size but up to half the size of the nucleus and surrounded by a clear halo, are indicative of poxvirus
166 infection. Syncytia formation is not a feature of capripoxvirus infection. If the CPE is due to
167 capripoxvirus infection of the cell culture, it can be prevented or delayed by inclusion of specific anti-
168 capripoxvirus serum in the medium; this provides a presumptive identification of the agent. Some
169 strains of capripoxvirus have been adapted to grow on African green monkey kidney (Vero) cells, but
170 these cells are not recommended for primary isolation.

171 1.3. Electron microscopy

172 The characteristic poxvirus virion can be visualised using a negative-staining preparation technique
173 followed by examination with an electron microscope. There are many different negative-staining
174 protocols, an example is given below:

175 Material from the original tissue suspension is prepared for transmission electron microscope
176 examination, prior to centrifugation, by floating a 400-mesh hexagon electron microscope grid, with
177 piliform-carbon substrate activated by glow discharge in pentylamine vapour, on to a drop of the
178 suspension placed on parafilm or a wax plate. After 1 minute, the grid is transferred to a drop of
179 Tris/EDTA buffer, pH 7.8, for 20 seconds and then to a drop of 1% phosphotungstic acid, pH 7.2, for
180 10 seconds. The grid is drained using filter paper, air-dried and placed in the electron microscope.
181 The capripoxvirus virion is brick shaped, covered in short tubular elements and measures
182 approximately 290 × 270 nm. A host-cell-derived membrane may surround some of the virions, and
183 as many as possible should be examined to confirm their appearance (Kitching & Smale, 1986).

184 The virions of capripoxvirus are indistinguishable from those of orthopoxvirus, but, apart from
185 *Vaccinia* virus, no orthopoxvirus causes lesions in sheep and goats. However, capripoxvirus is
186 distinguishable from the virions of parapoxvirus, that cause contagious pustular dermatitis, as they
187 are smaller, oval in shape, and each is covered in a single continuous tubular element, which appears
188 as striations over the virion.

189 1.4. Histopathology

190 Material for histopathology and immunohistochemistry should be prepared by standard techniques
191 (Parvin *et al.*, 2022). Following preparation, and staining with haematoxylin and eosin (H&E), and
192 mounting of the formalin-fixed biopsy material, a number of sections should be examined by light
193 microscopy. On histological examination, the most striking aspects of acute-stage skin lesions are a
194 massive cellular infiltrate, vasculitis and oedema. Early lesions are characterised by marked
195 perivascular cuffing. Initially infiltration is by macrophages, neutrophils and occasionally eosinophils,
196 and as the lesion progresses, by more macrophages, lymphocytes and plasma cells. A characteristic
197 feature of all capripoxvirus infections is the presence of variable numbers of 'sheep pox cells' in the
198 dermis. These sheep pox cells can also occur in other organs where microscopic lesions of sheep
199 and goat pox are present. These cells are large, stellate cells with eosinophilic, poorly defined
200 intracytoplasmic inclusions and vacuolated nuclei. Vasculitis is accompanied by thrombosis and
201 infarction, causing oedema and necrosis. Epidermal changes consist of acanthosis, parakeratosis
202 and hyperkeratosis. Changes in other organs are similar, with a predominant cellular infiltration and
203 vasculitis. Lesions in the upper respiratory tract are characterised by ulceration.

204 Immunohistochemistry will show capripox virus antigen infiltrated macrophages throughout the
205 subcutis. The capripox virus antigen can occasionally be detected in hair follicle epithelial cells, the
206 endothelium and smooth muscle cells of the blood vessels, and histiocytic cells (Parvin *et al.*, 2022).

207

1.5. Immunological methods

208

1.5.1. Fluorescent antibody tests

209

Capripoxvirus antigen can also be identified on infected cover-slips or tissue culture slides using fluorescent antibody tests. Cover-slips or slides should be washed and air-dried and fixed in cold acetone for 10 minutes. The indirect test using immune sheep or goat sera is subject to high background colour and nonspecific reactions. However, a direct conjugate can be prepared from sera from convalescent sheep or goats or from rabbits hyperimmunised with purified *Capripoxvirus*. Uninfected tissue culture should be included as a negative control because cross-reactions, due to antibodies to cell culture antigens, can cause problems. The fluorescent antibody tissue section technique has also been used on cryostat-prepared slides.

217

1.6. Nucleic acid recognition methods

218

Amplification methods for detection of the viral DNA genome are specific to the genus *Capripoxvirus* DNA and both specific and sensitive for detection throughout the course of disease, including before and after the emergence of antibody responses. These methods include conventional PCR, real-time PCR, and most recently loop-mediated isothermal amplification (LAMP). Nucleic acid recognition methods can be used to detect the *Capripoxvirus* genome in biopsy, swab, blood, semen or tissue culture samples. It is important that nucleic acid extraction and PCR amplification methods are validated for the sample matrix being tested.

225

1.6.1. Conventional PCR methods

226

Several conventional PCR methods have been reported with varying specificity for capripoxviruses in general, SPPV, or GTPV (Heine *et al.*, 1999; Ireland & Binopal, 1998; Zro *et al.*, 2014a). A conventional PCR assay that differentiates GTPV and LSDV from SPPV has been described (Lamien *et al.*, 2011a). Conventional PCR methods are particularly useful for obtaining sufficient genetic material necessary for species identification by subsequent sequence and phylogenetic analysis (Le Goff *et al.*, 2009).

232

The conventional gel-based PCR method described below is a simple, fast and sensitive method for the detection of capripoxvirus genome in EDTA blood, semen or tissue culture samples (Tuppurainen *et al.*, 2005).

234

235

Test procedure

236

The extraction method described below can be replaced using commercially available DNA extraction kits.

237

238

i) Freeze and thaw 200 µl of blood in EDTA, semen or tissue culture supernatant and suspend in 100 µl of lysis buffer containing 5 M guanidine thiocyanate, 50 mM potassium chloride, 10 mM Tris/HCl (pH 8); and 0.5 ml Tween 20.

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ii) Cut skin and other tissue samples into fine pieces using a sterile scalpel blade and forceps. Grind with a pestle in a mortar. Suspend the tissue samples in 800 µl of the above mentioned lysis buffer.

242

243

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iii) Add 2 µl of proteinase K (20 mg/ml) to blood samples and 10 µl of proteinase K (20 mg/ml) to tissue samples. Incubate at 56°C for 2 hours or overnight, followed by heating at 100°C for 10 minutes. Add phenol:chloroform:isoamylalcohol (25:24:1 [v/v]) to the samples in a 1:1 ratio. Vortex and incubate at room temperature for 10 minutes. Centrifuge the samples at 16,060 g for 15 minutes at 4°C. Carefully collect the upper, aqueous phase (up to 200 µl) and transfer into a clean 2.0 ml tube. Add two volumes of ice cold ethanol (100%) and 1/10 volume of 3 M sodium acetate (pH 5.3). Place the samples at -20°C for 1 hour. Centrifuge again at 16,060 g for 15 minutes at 4°C and discard the supernatant. Wash the pellets with ice cold 70% ethanol (100 µl) and centrifuge at 16,060 g for 1 minute at 4°C. Discard the supernatant and dry the pellets thoroughly. Suspend the pellets in 30 µl of nuclease-free water and store immediately at

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255 -20°C (Tuppurainen *et al.*, 2005). Alternatively a column-based extraction kit may be
256 used.

257 iv) The primers for this PCR assay were developed from the gene encoding the viral
258 attachment protein. The size of the expected amplicon is 192 bp (Ireland & Binopal,
259 1998). The primers have the following gene sequences:

260 Forward primer 5'-TCC-GAG-CTC-TTT-CCT-GAT-TTT-TCT-TAC-TAT-3'

261 Reverse primer 5'-TAT-GGT-ACC-TAA-ATT-ATA-TAC-GTA-AAT-AAC-3'.

262 v) DNA amplification is carried out in a final volume of 50 µl containing: 5 µl of 10 × PCR
263 buffer, 1.5 µl of MgCl₂ (50 mM), 1 µl of dNTP (10 mM), 1 µl of forward primer, 1 µl of
264 reverse primer, 1 µl of DNA template (~10 ng), 0.5 µl of *Taq* DNA polymerase and 39 µl
265 of nuclease-free water. The volume of DNA template required may vary and the volume
266 of nuclease-free water must be adjusted to the final volume of 50 µl.

267 vi) Run the samples in a thermal cycler as follows: 2 minutes at 95°C; then 45 seconds at
268 95°C, 50 seconds at 50°C and 1 minute at 72°C (34 cycles); 2 minutes at 72°C and hold
269 at 4°C until analysis.

270 vii) Mix 10 µl of each sample with loading dye and load onto a 1.5% agarose gel in TAE buffer
271 (Tris/acetate buffer containing EDTA). Load a parallel lane with a 100 bp DNA-marker
272 ladder. Electrophoretically separate the products using approximately 8–10 V/cm for 40–
273 60 minutes and visualise with a suitable DNA stain and transilluminator.

274 **1.6.2. Real-time PCR methods**

275 Several highly sensitive and specific fluorescent detection-based real-time PCR methods have
276 been developed and validated (Balinsky *et al.*, 2008; Bowden *et al.*, 2008; Das *et al.*, 2012;
277 Stubbs *et al.*, 2012). Each test detects a small conserved genetic locus within the capripoxvirus
278 genome, but these methods do not discriminate between SPPV, GTPV or LSDV. Real-time
279 PCR methods for direct capripoxvirus genotyping species differentiation without the need for
280 gene sequencing have been described (Haegeman *et al.*, 2013; Gelaye *et al.*, 2013; Lamien
281 *et al.*, 2011b; Wolff *et al.*, 2021).

282 The real-time PCR method described below is a rapid, sensitive and specific method for the
283 detection of the genomic DNA from SPPV, GTPV or LSDV. This assay will is not designed to
284 differentiate between the capripoxvirus species.

285 DNA extraction from blood, and tissue and semen

286 A number of DNA extraction kits are commercially available for the isolation extraction of
287 template DNA for real-time PCR. Manufacturer's instructions should always be consulted for
288 guidance on the appropriate method for the sample type being extracted followed while using
289 commercial extraction kits. WOA Reference Laboratories can be contacted for advice on
290 suitable commercial kits.

291 Real-time PCR

292 i) The real-time PCR method outlined below uses the primers and probe described by
293 Bowden *et al.* (2008). and further validated by Stubbs *et al.* (2012). Cycling conditions
294 and reagent concentrations can be altered to ensure optimal performance in individual
295 laboratories.

296 ii) Forward and reverse primers should be prepared at concentrations of 20 µM. A minor
297 grove binder (MGB) TaqMan hydrolysis probe should be prepared at a concentration of
298 10 µM.

299 Forward primer: 5'-AAA-ACG-GTA-TAT-GGA-ATA-GAG-TTG-GAA-3'

300 Reverse primer: 5'-AAA-TGA-AAC-CAA-TGG-ATG-GGA-TA-3'

301 Probe: 5'-FAM-TGG-CTC-ATA-GAT-TTC-CT-MGB-3'

- 302 iii) Mastermix is prepared by combining 10 µl of 2 × real-time PCR mastermix with 0.4 µl of
303 forward primer, 0.4 µl of reverse primer, 0.5 µl of probe and 6.7 µl of RNase free water
304 per reaction.
- 305 iv) Add 2 µl of extracted DNA to 18 µl of mastermix in a 96-well PCR plate or PCR strip and
306 perform real-time PCR according to the example given below or similar method:
- 307 v) 95°C for 10 minutes followed by 45 cycles of 95°C for 15 seconds, 60°C for 60 seconds.
308 Fluorescence detection should be performed at the end of each cycle.
- 309 vi) Following completion of the real-time PCR, a cycle threshold (C_T) should be set. Samples
310 with C_T values less than 35 are considered positive. Samples with a C_T value greater than
311 35 but less than 45 are considered inconclusive and require further investigation.
312 Samples which do not yield a C_T value, i.e. the amplification curve does not cross the
313 threshold, are considered negative.

314 1.6.3. Isothermal genome amplification

315 Molecular tests using ~~loop-mediated isothermal amplification (LAMP)~~ to detect capripoxvirus
316 genomes are reported to provide sensitivity and specificity similar to real-time PCR with a
317 simpler method and at lower cost (Das *et al.*, 2012; Murray *et al.*, 2013). Field validation of the
318 Das *et al.* (2012) LAMP ~~method assay~~ has been further reported ~~by~~ (Omoga *et al.*, 2016) and
319 a combination of this universal capripoxvirus test with two additional LAMP assays was
320 reported ~~to show utility in discriminating between to differentiate~~ GTPV ~~and from~~ SPPV (Zhao
321 *et al.*, 2014).

322 2. Serological tests

323 Detectable levels of antibodies develop 1 week after the animal shows clinical signs. The highest antibody
324 levels are detected within 1–2 months after infection is detected.

325 2.1. Virus neutralisation

326 A test serum can either be titrated against a constant titre of capripoxvirus (100 TCID₅₀ [50% tissue
327 culture infective dose]) or a standard capripoxvirus strain can be titrated against a constant dilution
328 of test serum in order to calculate a neutralisation index. Because of the variable sensitivity of tissue
329 culture to capripoxvirus, and the consequent difficulty of ensuring the use of 100 TCID₅₀, the
330 neutralisation index is the preferred method, although it does require a larger volume of test sera.
331 The test is described using 96-well flat-bottomed tissue culture grade microtitre plates, but it can be
332 performed equally well in tissue culture tubes with the appropriate changes to the volumes used,
333 although it is more difficult to read an end-point in tubes. ~~The use of Vero cells in the virus~~
334 ~~neutralisation test has been reported to give more consistent results (Kitching & Taylor, 1985).~~

335 2.1.1. Test procedure

- 336 i) Test sera including a negative and a positive control are diluted 1/5 in Eagle's/HEPES
337 (N-2-hydroxyethylpiperazine, N-2-ethanesulphonic acid) and inactivated at 56°C for
338 30 minutes.
- 339 ii) Next, 50 µl of the first inactivated serum is added to columns 1 and 2, rows A to H of the
340 microtitre plate. The second serum is placed in columns 3 and 4, the third in columns
341 5 and 6, the positive control serum is placed in columns 7 and 8, the negative control
342 serum is placed in columns 9 and 10, and 50 µl of Eagle's/HEPES without serum is placed
343 in columns 11 and 12 and to all wells of row H.
- 344 iii) A reference strain of capripoxvirus, usually a vaccine strain known to grow well in tissue
345 culture, with a titre of over log₁₀ 6 TCID₅₀ per ml is diluted in Eagle's/HEPES in bijoux
346 bottles to give a log dilution series of log₁₀ 5.0; 4.0; 3.5; 3.0; 2.5; 2.0; 1.5 TCID₅₀ per ml
347 (equivalent to log₁₀ 3.7; 2.7; 2.2; 1.7; 1.2; 0.7; 0.2 TCID₅₀ per 50 µl).
- 348 iv) Starting with row G and the most diluted virus preparation, 50 µl of virus is added to each
349 well in that row. This is repeated with each virus dilution, the highest titre virus dilution
350 being placed in row A.

- 351 v) The plates are covered and incubated for 1 hour at 37°C.
- 352 vi) ~~LT cells are~~ An appropriate cell suspension (such as MDBK cells) is prepared from
353 pregrown monolayers as a suspension of 10⁵ cells/ml in Eagle's medium containing
354 antibiotics and 2% fetal calf serum. Following incubation of the microtitre plates, 100 µl of
355 cell suspension is added to all the wells, except wells H11 and H12, which serve as control
356 wells for the medium. The remaining wells of row H are cell and serum toxicity controls.
- 357 vii) The microtitre plates are covered and incubated at 37°C for 9 days.
- 358 viii) Using an inverted microscope, the monolayers are examined daily starting at day 4 for
359 evidence of CPE. There should be no CPE in the cells of row H. Using the 0240 KSGP
360 vaccine strain of capripoxvirus, the final reading is taken on day 9, and the titre of virus in
361 each duplicate titration is calculated according to the Kärber method. If left longer, there
362 is invariably a 'breakthrough' of virus in which virus that was at first neutralised appears
363 to disassociate from the antibody.
- 364 ix) *Interpretation of the results:* The neutralisation index is the log titre difference between
365 the titre of the virus in the negative serum and in the test serum. An index of ≥1.5 is
366 positive. The test can be made more sensitive if serum from the same animal is examined
367 before and after infection. Because immunity to capripoxvirus is predominantly cell
368 mediated, a negative result, particularly following vaccination in which the response is
369 necessarily mild, does not imply that the animal from which the serum was taken is not
370 protected.

371 ~~A constant virus/varying serum method has been described using serum dilutions in the~~
372 ~~range 1/5 to 1/500 and fetal calf muscle cells. Because these cells have a lower sensitivity~~
373 ~~to capripoxvirus than LT cells, the problem of virus 'breakthrough' is overcome.~~

374 2.2. Indirect fluorescent antibody test

375 Capripoxvirus-infected tissue culture grown on flying cover-slips or tissue culture microscope slides
376 can be used for the indirect fluorescent antibody test. Uninfected tissue culture control, and positive
377 and negative control sera, should be included in the test. The infected and control cultures are fixed
378 in acetone at -20°C for 10 minutes and stored at 4°C. Dilutions of test sera are made in PBS, starting
379 at 1/5, and positives are identified using an anti-sheep gamma-globulin conjugated with fluorescein
380 isothiocyanate (Davies & Otema, 1978). Cross-reactions can occur with orf, bovine papular stomatitis
381 virus and perhaps other poxviruses.

382 2.3. Western blot analysis

383 Western blotting of test sera against capripoxvirus-infected cell lysate provides a sensitive and
384 specific system for the detection of antibody to capripoxvirus structural proteins, although the test is
385 expensive and difficult to carry out (Chand *et al.*, 1994).

386 2.4. Enzyme-linked immunosorbent assay

387 ~~No validated ELISA is available for the serological diagnosis of SPP or GTP.~~

388 Both in-house and commercial enzyme-linked immunosorbent assay (ELISAs) are available, but
389 these tests cannot discriminate between antibodies to different capripoxviruses (LSDV or
390 SPPV/GTPV).

C. REQUIREMENTS FOR VACCINES

[THIS SECTION IS UNDER REVIEW IN THE 2024/2025 REVIEW CYCLE]

1. Background

1.1. Rationale and intended use of the product

A variety of attenuated live and inactivated capripoxvirus vaccines has been used to provide protection against sheeppox and goatpox. All strains of capripoxvirus of ovine, caprine or bovine origin examined so far share a major neutralising site, so that animals recovered from infection with one strain are resistant to infection with any other strain (Capstick, 1961). Consequently, it is possible to use a single strain of capripoxvirus to protect both sheep and goats against all field strains of virus, regardless of whether their origin was in Asia or Africa (Kitching *et al.*, 1986; Kitching & Taylor, 1985). However, field evidence suggests some strains are quite host-specific and are used only in sheep against SPPV and only in goat against GTPV.

A number of strains of capripoxvirus have had widespread use as live vaccines (Davies & Mbugwa, 1985), for example the Romanian and RM-65 strains used mainly in sheep and the Mysore and Gorgan strains used in goats. The real identity of the commonly used Kenyan sheep and goat pox vaccine virus (KSGP) 0240 was recently shown to be actually LSDV (Tuppurainen *et al.*, 2014). Virus strain identity and attenuation properties must be ascertained and taken into consideration when selecting vaccine strains for use in cattle, sheep and goats. The protective dose depends on the vaccine strain used. Immunity in sheep and goats against capripoxvirus following vaccination with the 0240 strain lasts over a year and the Romanian strain gave protection for at least 30 months.

Killed vaccines produced from tissue culture contain only the intracellular mature virion form of the virus, and lack the less robust but biologically crucial extracellular enveloped virion form. As a result, the vaccine does not stimulate immunity against the extracellular enveloped virion, resulting in poor protection. Killed capripoxvirus vaccines provide, at best, only temporary protection.

2. Outline of production and minimum requirements for conventional vaccines

General requirements set for the facilities used for the production of vaccines and for the documentation and record keeping throughout the whole manufacturing process are described in Chapter 1.1.8 *Principles of veterinary vaccine production*. The documentation should include the standard operating procedures (SOP) for the method of manufacture and each step for the testing of cells and reagents used in the process, each batches and the final product.

2.1. Characteristics of the seed

2.1.1. Biological characteristics

A strain of capripoxvirus used for vaccine production must be accompanied by a history describing its origin and tissue culture or animal passage. It must be safe to use in all breeds of sheep and goats for which it is intended, including pregnant and young animals. It must be non-transmissible, remain attenuated after further tissue culture passage, and provide complete protection against challenge with virulent field strains for a minimum of 1 year. A quantity of master seed vaccine virus should be prepared and stored in order to provide a consistent working seed for regular vaccine production.

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

Each master seed must be tested to ensure its identity and shown to be free from adventitious viruses, in particular pestiviruses, such as border disease and bovine viral diarrhoea virus, and free from contamination with bacteria, fungi and/or mycoplasmas. The general procedures for sterility or purity tests are described in chapter 1.1.9. The master seed must also be safe and produce no clinical reaction in all breeds of sheep or goats when given by the recommended route and stimulate complete immunity to capripoxvirus in all breeds of sheep and goats for at

437 least 1 year. The necessary safety and potency tests are described in Section C.2.2.4 *Final*
438 *product batch tests*.

439 **2.2. Method of manufacture**

440 The method of manufacture should be documented as the Outline of Production.

441 **2.2.1. Procedure**

442 Vaccine seed should be lyophilised and stored in 2 ml vials at -20°C . It may be stored wet at
443 -20°C , but when wet, is more stable at -70°C or lower. The virus should be cultured in primary
444 or secondary LT or LK cells of wool sheep origin for maximum yield. Vero cells may also be
445 used with suitably adapted strains.

446 Vaccine batches are produced on fresh monolayers of secondary LT or primary LK cells. A
447 vial of seed virus is reconstituted with GMEM or another appropriate medium and inoculated
448 on to an LT or LK monolayer that has been previously washed with warm PBS, and allowed
449 to adsorb for 15 minutes at 37°C before being overlaid with additional GMEM. After 4–6 days,
450 there will be extensive (80–90%) CPE. The culture should be examined for any evidence of
451 nonspecific CPE, medium cloudiness or change in medium pH. The culture is freeze–thawed
452 three times, the suspension removed and centrifuged at 600 **g** for 20 minutes. A second
453 passage may be required to produce sufficient virus for a production batch. Live vaccine may
454 be produced on roller bottles.

455 The procedure is repeated and the harvests from individually numbered flasks are each mixed
456 separately with an equal volume of sterile and chilled 5% lactalbumin hydrolysate and 10%
457 sucrose, and transferred to individually numbered bottles for storage at -20°C . Prior to
458 storage, 0.2 ml is removed from each bottle for sterility control. An additional 0.2 ml is removed
459 for virus titration; 2 ml pools composed of 0.2 ml samples taken from ten bottles are used. A
460 written record of all the procedures must be kept for all vaccine batches.

461 Inactivated vaccines are produced, usually from unattenuated field strains of capripoxvirus,
462 grown in tissue culture as described above, inactivated with 0.03% formaldehyde, and mixed
463 with an equal volume of alhydrogel as adjuvant. Formaldehyde is no longer considered to be
464 a suitable inactivant for certain viral vaccines because its mode of action cannot be guaranteed
465 to be totally effective in inactivating all the live virus. This has not been fully investigated for
466 capripoxvirus.

467 **2.2.2. Requirements for substrate and media**

468 The specification and source of all ingredients used in the manufacturing procedure should be
469 documented and the freedom from extraneous agents: bacteria, fungi, mycoplasma and any
470 other viruses should be tested. The detailed testing procedure is described in the chapter
471 1.1.9. The use of antibiotics must meet the requirements of the licensing authority.

472 **2.2.3. In-process controls**

473 **i) Cells**

474 Cells should be obtained from the testis or kidney of a healthy young lamb from a scrapie-
475 free flock of a wool sheep breed. During cultivation, cells must be observed for any
476 evidence of CPE, and for normal morphology (predominantly fibroblastic). They can
477 usually be passaged successfully up to ten times. When used for vaccine production,
478 uninfected control cultures should be grown in parallel and maintained for at least three
479 additional passages for further observation. They should be checked for the presence of
480 noncytopathic strains of bovine virus diarrhoea or border disease viruses by
481 immunofluorescence or immunoperoxidase techniques. If possible, cells should be
482 prepared and screened prior to vaccine production and stocked in 1–2 ml aliquots
483 containing 2×10^7 cells/ml in sterile 10% DMSO (dimethyl sulphoxide) and 90% FBS
484 (fetal bovine serum) solution stored in liquid nitrogen.

- 485 ii) Serum
486 Bovine serum used in the growth or maintenance medium must be free from transmissible
487 spongiform encephalopathies (TSEs) and antibody to capripoxvirus, and tested for
488 contamination with pestivirus or any other viruses, extraneous bacteria, mycoplasma or
489 fungi.
- 490 iii) Medium
491 Medium must be tested free from contamination with pestivirus or any other viruses,
492 extraneous bacteria, mycoplasma or fungi.
- 493 iv) Virus
494 Seed virus and final vaccine must be titrated in tissue culture tubes or microtitre plates.
495 Vaccine samples must be examined for the presence of adventitious viruses including
496 cytopathic and noncytopathic strains of pestivirus, and should be mixed with a high-titre
497 capripoxvirus-immune serum that has tested negative for antibody to pestivirus to prevent
498 the vaccine virus itself interfering with the test. The vaccine bulk can be held at -20°C or
499 below until all sterility tests and titrations have been completed, at which time it should be
500 freeze-dried in 1 ml aliquots in vials sufficient for 100 doses. The vaccine harvest diluted
501 with lactalbumin hydrolysate and sucrose should have a minimum titre \log_{10} 4.5 TCID₅₀
502 per ml after freeze-drying, equivalent to a field dose of \log_{10} 2.5 TCID₅₀. A further titration
503 is carried out on five randomly chosen vials of the freeze-dried preparation to confirm the
504 titre.

505 2.2.4. Final product batch tests

- 506 i) Sterility/purity
507 Tests for sterility and freedom from contamination of biological materials intended for
508 veterinary use may be found in chapter 1.1.9.
- 509 ii) Safety
510 The safety studies should be demonstrated by statistically valid vaccination studies using
511 seronegative young sheep and goats of known susceptibility to capripox virus. The
512 procedure described is suitable for vaccine strains such as 0240 that are equally
513 immunogenic in both sheep and goats. The choice of target animal should be adapted for
514 strains with a more restricted host preference.
- 515 iii) Potency
516 Potency tests must be undertaken if the minimum immunising dose of the virus strain is
517 not known. This is usually carried out by comparing the titre of a virulent challenge virus
518 on the flanks of vaccinated and control animals. Following vaccination, the flanks of at
519 least three animals and three controls are shaved of wool or hair. \log_{10} dilutions of the
520 challenge virus are prepared in sterile PBS and six dilutions are inoculated intradermally
521 (0.1 ml per inoculum) along the length of the flank; four replicates of each dilution are
522 inoculated down the flank. An oedematous swelling will develop at possibly all
523 24 inoculation sites on the control animals, although preferably there will be little or no
524 reaction at the four sites of the most dilute inocula. The vaccinated animals should
525 develop an initial hypersensitivity reaction at sites of inoculation within 24 hours, which
526 should quickly subside. Small areas of necrosis may develop at the inoculation site of the
527 most concentrated challenge virus. The macule/papule is measured at between 8 and 10
528 days post-challenge. The titre of the challenge virus is calculated for the vaccinated and
529 control animals; a difference of \log_{10} titre > 2.5 is taken as evidence of protection.

530 **2.3. Requirements for authorisation**

531 **2.3.1. Safety requirements**

532 i) Target and non-target animal safety

533 The vaccine must be safe to use in all breeds of sheep and goats for which it is intended,
534 including young and pregnant animals. It must also be non-transmissible, remain
535 attenuated after further tissue culture passage.

536 Safety tests should be carried out on the final product of each batch as described in
537 Section C.2.2.4.

538 The safety of the vaccine in non-target animals must have been demonstrated using mice
539 and guinea-pigs as described in Section C.2.2.4. There should be no evidence of
540 pathology caused by the vaccine.

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

542 The selected final vaccine should not revert to virulence during a further passages in
543 target animals.

544 iii) Environmental consideration

545 Attenuated vaccine should not be able to perpetuate autonomously in cattle, sheep or
546 goat populations. Vaccines using the 0240 strain should not be used in *Bos taurus*
547 breeds. Strains of capripoxvirus are not a hazard to human health. There are no
548 precautions other than those described above for sterility and freedom from adventitious
549 agents.

550 **2.3.2. Efficacy requirements**

551 i) For animal production

552 The efficacy of the vaccine must be demonstrated in vaccination challenge experiment
553 under laboratory conditions. As described in Section C.2.2.4.

554 Once the potency of the particular strain being used for vaccine production has been
555 determined in terms of minimum dose required to provide immunity, it is not necessary to
556 repeat this on the final product of each batch, provided the titre of virus present has been
557 ascertained.

558 ii) For control and eradication

559 Vaccination is the only effective way to control the sheep pox and goat pox outbreaks in
560 endemic countries. Unfortunately, currently no marker vaccines allowing the
561 differentiation of infected from vaccinated animals are available.

562 Immunity to virulent field virus following vaccination of sheep or goats with the 0240 strain
563 lasts over 1 year, and protection against generalised infection following intradermal
564 challenge lasts at least 3 years and is effective lifelong. The duration of immunity
565 produced by other vaccine strains should be ascertained in both sheep and goats by
566 undertaking controlled trials in an environment in which there is no possibility of field
567 strains of capripoxvirus confusing the results. The inactivated vaccines provide immunity
568 for less than 1 year, and for the reasons given at the beginning of this section, may not
569 give immunity to the form of capripoxvirus usually associated with natural transmission.

570 **2.3.3. Stability**

571 All vaccines are initially given a shelf-life of 24 months before expiry. Real-time stability studies
572 are then conducted to confirm the appropriateness of the expiry date. Multiple batches of the
573 vaccine should be re-titrated periodically throughout the shelf-life to determine the vaccine
574 variability.

575 Properly freeze-dried preparations of capripox vaccine, particularly those that include a
576 protectant, such as sucrose and lactalbumin hydrolysate, are stable for over 25 years when
577 stored at –20°C and for 2–4 years when stored at 4°C. There is evidence that they are stable
578 at higher temperatures, but no long-term controlled experiments have been reported. The
579 inactivated vaccines must be stored at 4°C, and their shelf- life is usually given as 1 year.

580 No preservatives other than a protectant, such as sucrose and lactalbumin hydrolysate, are
581 required for the freeze-dried preparation.

582 **3. Vaccines based on biotechnology**

583 **3.1. Vaccines available and their advantages**

584 Currently, no recombinant vaccines for capripoxviruses are commercially available. However, a new
585 generation of capripox vaccines is being developed that uses the capripoxvirus genome as a vector
586 for the genes of other ruminant pathogens such as peste des petits ruminants (PPR) virus (Berhe *et*
587 *al.*, 2003; Tuppurainen *et al.*, 2014).

588 **3.2. Special requirements for biotechnological vaccines, if any**

589 Not applicable.

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671 **NB:** There are WOAHP Reference Laboratories for sheep pox and goat pox (please consult the WOAHP Web
672 site:

673 <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>).

674 Please contact the WOAHP Reference Laboratories for any further information on
675 diagnostic tests, reagents and vaccines for sheep pox and goat pox

676 **NB:** FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2017.

Annexe 16. Chapter 3.9.1. 'African swine fever (infection with African swine fever virus)'

SECTION 3.9.

SUIDAE

CHAPTER 3.9.1.

AFRICAN SWINE FEVER (INFECTION WITH AFRICAN SWINE FEVER VIRUS)

SUMMARY

African swine fever (ASF) is an infectious disease of domestic and wild pigs of all breeds and ages, caused by ASF virus (ASFV). The clinical syndromes vary from peracute, acute, subacute to chronic, depending on the virulence of the virus. Acute disease is characterised by high fever, haemorrhages in the reticuloendothelial system, and a high mortality rate. Soft ticks of the Ornithodoros genus, especially O. moubata and O. erraticus, have been shown to be both reservoirs and transmission vectors of ASFV. The virus is present in tick salivary glands and passed to new hosts (domestic or wild suids) when feeding. It can be transmitted sexually between ticks, transovarially to the eggs, or transtadially throughout the tick's life.

ASFV is the only member of the Asfarviridae family, genus Asfivirus.

Laboratory diagnostic procedures for ASF fall into two groups: detection of the virus and serology. The selection of the tests to be carried out depends on the disease situation and laboratory diagnostic capacity in the area or country.

Identification of the agent: *Laboratory diagnosis must be directed towards isolation of the virus by inoculation of pig leukocyte or bone marrow cultures, the detection of antigen in smears or cryostat sections of tissues by fluorescent antibody test and/or the detection of genomic DNA by the polymerase chain reaction (PCR) or real-time PCR. The PCRs are excellent, highly sensitive, specific and rapid techniques for ASFV detection and are very useful under a wide range of circumstances. They are especially useful if the tissues are unsuitable for virus isolation and antigen detection. In doubtful cases, the material is passaged in leukocyte cell cultures and the procedures described above are repeated.*

Serological tests: *Pigs that survive natural infection usually develop antibodies against ASFV from 7–10 days post-infection and these antibodies persist for long periods of time. Where the disease is endemic, or where a primary outbreak is caused by a strain of low or moderate virulence, the investigation of new outbreaks should include the detection of specific antibodies in serum or extracts of the tissues submitted. A variety of methods such as the enzyme-linked immunosorbent assay (ELISA), the indirect fluorescent antibody test*

35 (IFAT), the indirect immunoperoxidase test (IPT), and the immunoblotting test (IBT) is
36 available for antibody detection.

37 **Requirements for vaccines:** ~~At present, there is no vaccine for ASF. Commercially~~
38 ~~produced modified live virus vaccines are available and licenced under field evaluation in~~
39 ~~some countries.~~

40 A. INTRODUCTION

41 The current distribution of African swine fever (ASF) extends across more than 50 countries in three
42 continents (Africa, Asia and Europe). Several incursions of ASF out of Africa were reported between
43 the 1960s and 1970s. In 2007, ASF was introduced into Georgia, from where it spread to neighbouring
44 countries including the Russian Federation. From there ASF spread to eastern European countries
45 extending westwards and reaching the European Union in 2014. Further westward and southern spread
46 in Europe has occurred since that time. In all these countries, both hosts – domestic pig and wild boar
47 – were affected by the disease. In August 2018, the People’s Republic of China reported its first
48 outbreak of ASF and further spread in Asia has occurred. ASF was identified on the island of Hispaniola
49 (Haiti and the Dominican Republic) in 2021. See WAHIS (<https://wahis.woah.org/#/home>) for recent
50 information on distribution at the country level.

51 ASF virus (ASFV) is a complex large, enveloped DNA virus with icosahedral morphology. It is currently
52 classified as the only member of the *Asfviridae* family, genus *Asfivirus* (Dixon *et al.*, 2005). More than
53 60 structural proteins have been identified in intracellular virus particles (200 nm) (Alejo *et al.*, 2018).
54 More than a hundred infection-associated proteins have been identified in infected porcine
55 macrophages, and at least 50 of them react with sera from infected or recovered pigs (Sánchez-
56 Vizcaíno & Arias, 2012). The ASFV double-stranded linear DNA genome comprises between 170 and
57 193 kilobases (kb) and contains between 150 and 167 open reading frames with a conserved central
58 region of about 125 kb and variable ends. These variable regions encode five multigene families that
59 contribute to the variability of the virus genome. The complete genomes of several ASFV strains have
60 been sequenced (Bishop *et al.*, 2015; Chapman *et al.*, 2011; de Villiers *et al.*, 2010; Portugal *et al.*,
61 2015). Different strains of ASFV vary in their ability to cause disease, but at present there is only one
62 recognised serotype of the virus detectable by antibody tests.

63 The molecular epidemiology of the disease is investigated by sequencing of the 3’ terminal end of the
64 B646L open reading frame encoding the p72 protein major capsid protein, which differentiates up to 24
65 distinct genotypes (Achenbach *et al.*, 2017; Boshoff *et al.*, 2007; Quembo *et al.* 2018). To distinguish
66 subgroups among closely related ASFV, sequence analysis of the tandem repeat sequences (TRS),
67 located in the central variable region (CVR) within the B602L gene (Gallardo *et al.*, 2009; Lubisi *et al.*,
68 2005; Nix *et al.*, 2006) and in the intergenic region between the I73R and I329L genes, at the right end
69 of the genome (Gallardo *et al.*, 2014), is undertaken. Several other gene regions such as the E183L
70 encoding p54 protein, the CP204L encoding p30 protein, and the protein encoded by the EP402R gene
71 (CD2v), have been proved as useful tools to analyse ASFVs from different locations and hence track
72 virus spread.

73 ASF viruses produce a range of syndromes varying from peracute, acute to chronic disease and
74 subclinical infections. Pigs are the only domestic animal species that is naturally infected by ASFV.
75 European wild boar and feral pigs are also susceptible to the disease, exhibiting clinical signs and
76 mortality rates similar to those observed in domestic pigs. In contrast African wild pigs such as warthogs
77 (*Phacochoerus aethiopicus*), bush pigs (*Potamochoerus porcus*) and giant forest hogs (*Hylochoerus*
78 *meinertzhageni*) are resistant to the disease and show few or no clinical signs. These species of wild
79 pig act as reservoir hosts of ASFV in Africa (Costard *et al.*, 2013; Sánchez-Vizcaíno *et al.*, 2015).

80 The incubation period is usually 4–19 days. The more virulent strains produce peracute or acute
81 haemorrhagic disease characterised by high fever, loss of appetite, haemorrhages in the skin and
82 internal organs, and death in 4–10 days, sometimes even before the first clinical signs are observed.
83 Case fatality rates may be as high as 100%. Less virulent strains produce mild clinical signs – slight
84 fever, reduced appetite and depression – which can be readily confused with many other conditions in
85 pigs and may not lead to suspicion of ASF. Moderately virulent strains are recognised that induce
86 variable disease forms, ranging from acute to subacute. Low virulence, non-haemadsorbing strains can

87 produce subclinical non-haemorrhagic infection and seroconversion, but some animals may develop
88 discrete lesions in the lungs or on the skin in areas over bony protrusions and other areas subject to
89 trauma. Animals that have recovered from either acute, subacute or chronic infections may potentially
90 become persistently infected, acting as virus carriers. The biological basis for the persistence of ASFV
91 is still not well understood, nor it is clear what role persistence plays in the epidemiology of the disease.

92 ASF cannot be differentiated from classical swine fever (CSF) by either clinical or post-mortem
93 examination, and both diseases should be considered in the differential diagnosis of any acute febrile
94 haemorrhagic syndrome of pigs. Bacterial septicaemias may also be confused with ASF and CSF.
95 Laboratory tests are essential to distinguish between these diseases.

96 In countries free from ASF but suspecting its presence, the laboratory diagnosis must be directed
97 towards isolation of the virus by the inoculation of pig leukocyte or bone marrow cultures, the detection
98 of genomic DNA by polymerase chain reaction (PCR) or the detection of antigen in smears or cryostat
99 sections of tissues by direct fluorescent antibody test (FAT). Currently the PCR is the most sensitive
100 technique and can detect ASFV DNA from a very early stage of infection in tissues, ethylene diamine
101 tetra-acetic acid (EDTA)-blood and serum samples. The PCR is particularly useful if samples submitted
102 are unsuitable for virus isolation and antigen detection because they have undergone putrefaction. Pigs
103 that have recovered from acute, subacute or chronic infections usually exhibit a viraemia for several
104 weeks making the PCR test a very useful tool for the detection of ASFV DNA in pigs infected with low
105 or moderately virulent strains. Virus isolation by the inoculation of pig leukocyte or bone marrow cultures
106 and identification by haemadsorption tests (HAD) are recommended as a confirmatory test when ASF
107 is positive by other methods, particularly in the event of a primary outbreak or a case of ASF.

108 ~~As no vaccine is available, the presence of ASFV antibodies is indicative of previous infection and, as~~
109 ~~antibodies are produced from the first week of infection and persist for long periods, they are a good~~
110 ~~marker for the diagnosis of the disease, particularly in subacute and chronic forms.~~

111 Vaccines should be prepared in accordance with Chapter 1.1.8 *Principles of veterinary vaccine*
112 *production*. Current ASF modified live virus (MLVs) vaccines are based on the live virus that have been
113 naturally attenuated or attenuated by targeted genetic recombination through cell cultures (Gladue &
114 Borca, 2022). MLV production is based on a seed-lot system consistent with the *European*
115 *Pharmacopoeia* (11th edition) and that has been validated with respect to virus identity, sterility, purity,
116 potency, stability, safety and immunogenicity (including spread), non-transmissibility, stability and
117 immunogenicity. ASF MLV first generation vaccines – defined as those for which peer-reviewed
118 publications are in the public domain – should meet or exceed the minimum standards as described
119 below. Paramount Demonstration of acceptable safety and efficacy against the epidemiologically
120 relevant ASFV field strain(s) circulating in areas where the vaccine is intended for use are is required.
121 At the present time, a variety of mutants (Forth *et al.*, 2023) and recombinants (Zhao *et al.*, 2023) have
122 emerged globally, and the prevalence of these strains is increasing. In addition, there is a risk that
123 vaccine strains will recombine with circulating strains. These conditions should be taken into account in
124 vaccine development. acceptable efficacy should be shown against the B646L- (p72) genotype II
125 pandemic virus lineage currently circulating widely in domestic pigs and wild boar.

126 ASF MLV first generation vaccines allowing the differentiation of infected animals from vaccinated
127 animals (DIVA) by suitable methods (e.g. serology-based tests) are preferred. Demonstration of MLV
128 safety and efficacy in pigs at different growth stages (suckling piglets, nursery pigs, fattening pigs), the
129 safety in breeding-age boars, gilts and pregnant sows, and onset and duration of protective immunity,
130 are also preferred but are not required to meet the minimum standard. Additional data will likely be
131 required by Regulatory Authorities if these categories are included in the indications for the vaccine.
132 Details of the onset of immunity (the interval of time elapsed between vaccination and challenge if
133 protection is confirmed) and the duration of immunity (the time point at which vaccine-induced immunity
134 begins to decline and provides less protection) are also required to meet minimum standards.

135 ASF epidemiology is complex with different epidemiological patterns of infection occurring in Africa and
136 Europe. ASF occurs through transmission cycles involving domestic pigs, wild boar, wild African suids,
137 and soft ticks (Sánchez-Vizcaino *et al.*, 2015). In regions where *Ornithodoros* soft-bodied ticks are
138 present, the detection of ASFV in these reservoirs of infection contributes to a better understanding of
139 the epidemiology of the disease. This is of major importance in establishing effective control and
140 eradication programmes (Costard *et al.*, 2013).

141 ASF is not a zoonotic disease and does not affect public health (Sánchez-Vizcaíno *et al.*, 2009).

142 ASFV should be handled with an appropriate level of bio-containment, determined by risk analysis in
143 accordance with Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the*
144 *veterinary laboratory and animal facilities*.

145 C. REQUIREMENTS FOR VACCINES [UNDER REVIEW]

146 At present there is no commercially available vaccine for ASF. Commercially produced modified live
147 virus vaccines are being evaluated and licensed for field use.

148 1. Background

149 The ASF p72 genotype II strains (ASFV Georgia 2007/1 lineage) (NCBI, 2020) are recognised to be
150 the current highest global threat for domestic pig production worldwide (Penrith *et al.*, 2022). However,
151 genotype I attenuated strains and genotype I/II recombinant strains have been reported to be
152 circulating. In Africa, multiple genotypes are circulating.

153 Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of Veterinary*
154 *Vaccine Production*. Varying additional requirements relating to quality (including purity and potency),
155 safety, and efficacy will apply in particular countries or regions for manufacturers to comply with local
156 regulatory requirements.

157 Wherever live, virulent ASFV or ASF MLVs are stored, handled and disposed, the appropriate
158 biosecurity level, procedures and practices should be used. The ASF MLV vaccine production facility
159 should meet the requirements for containment outlined in Chapter 1.1.4 *Biosafety and biosecurity:*
160 *Standard for managing biological risk in the veterinary laboratory and animal facilities*.

161 An optimal ASF MLV first generation vaccine for the target host should have the following general
162 characteristics (minimum standards):

- 163 • Safe: demonstrate absence of persistent fever as defined below (see Section 2.3.2) and clinical
164 signs of acute or chronic ASF in vaccinated and in-contact animals, minimal and ideally no vaccine
165 virus transmission, and absence of an increase in virulence (genetic and phenotypic stability);
- 166 • Efficacious: protects against mortality, reduces acute disease (fever accompanied by the
167 appearance of clinical signs caused by ASF) and reduces vertical (boar semen and placental) and
168 horizontal disease transmission;
- 169 • Quality – purity: free from wild-type ASFV and extraneous microorganisms that could adversely
170 affect the safety, potency or efficacy of the product;
- 171 • Quality – potent-stability: the log₁₀-virus titre maintained throughout the vaccine shelf life that
172 guarantees the efficacy demonstrated by the established minimum immunising (protective) dose.
- 173 • Identity-Vaccine matching: based on the capacity to protect against the ASFV B646L (p72)
174 genotype II pandemic strain or other p72 genotypes of recognised epidemiologic importance.

175 Vaccine production should be carried out using a validated, controlled and consistent manufacturing
176 process.

177 ASF MLV first generation vaccines must be safe (i.e. an acceptable safety profile) for non-target species
178 and the environment in general.

179 Ideally, ASF MLV first generation vaccines that meet the minimum standards should also fulfil the
180 following additional general characteristics: i) prevents acute and persistent (carrier state) disease;
181 ii) prevents horizontal and vertical disease transmission; iii) induces rapid protective immunity (e.g.
182 < 2 weeks); and iv) confers stable, life-long immunity.

183 Furthermore, ASF MLV second and future generation vaccines should meet the minimum safety and
184 efficacy standards as ASF MLV first generation vaccines, and ideally provide additional product profile
185 benefits, including but not limited to: i) contain a negative marker allowing the differentiation of infected
186 from vaccinated animals (DIVA) by reliable discriminatory tests such as serology-based tests; and ii)
187 confer broad range of protection against other p72 genotype field strains of varying virulence (low,
188 moderate, and high).

189 The majority of ASF global vaccine research groups and companies are currently focused on ASF MLV
190 first generation vaccine candidates that are safe and efficacious against ASF viruses belonging to the
191 ASFV p72 genotype II pandemic strain (ASFV Georgia 2007/1 lineage) (NCBI, 2020). More research is
192 needed to determine whether these genotype II-specific MLVs can effectively protect against newly
193 circulating variants of genotype II and recombinant strains.

194 Currently, two recombinant gene deleted MLV recombinant-vaccines (ASFV-G- Δ I177L and ASFV-G-
195 Δ MGF) have been licenced for field use in Vietnam for use in domestic pigs following supervised field
196 testing to evaluate the safety and effectiveness of several vaccine batches.

197 There are numerous, promising ASF MLV vaccine candidates targeting the p72 genotype II pandemic
198 strain under development, including:

- 199 • A naturally attenuated field strain (Lv17/WB/Rei1) (Barasona *et al.*, 2019) being developed as an
200 oral bait vaccine for wild boars;
- 201 • A laboratory thermo-attenuated field strain (ASFV-989) (Bourry *et al.*, 2022);
- 202 • Single gene-deleted, recombinant viruses (e.g. SY18 Δ I226R, ASFV-G- Δ A137R) (Gladue *et al.*,
203 2021; Zhang *et al.*, 2021);
- 204 • Double gene-deleted, recombinant viruses (e.g. ASFV-G- Δ 9GL/ Δ UK; ASFV-SY18- Δ CD2v/UK;
205 Arm- Δ CD2v- Δ A238L) (O'Donnell *et al.*, 2016; Pérez-Núñez *et al.*, 2022; Teklue *et al.*, 2020);
- 206 • Multiple gene-deleted, recombinant viruses (ASFV-G- Δ I177L/ Δ LVR; ASFV-G- Δ MGF; BA71 Δ CD2;
207 HLJ/18-7GD; ASFVGZ Δ I177L Δ CD2v Δ MGF) (Borca *et al.*, 2021; Chen *et al.*, 2020; Kitamura *et*
208 *al.*, 2023; Liu *et al.*, 2023; Monteagudo *et al.*, 2017; O'Donnell *et al.*, 2015).

209 Information regarding many of these MLV vaccine candidates can be found in a recent review
210 publication (Brake, 2022).

211 Different DIVA strategies using serological methods (e.g. ELISA) or genome detection methods (e.g.
212 differential real-time PCR) are not widely available for these ASF MLV first generation vaccine
213 candidates. Therefore, there is still room for improvement with respect to marker vaccines and their
214 companion diagnostic tests.

215 Inactivated (non-replicating) whole virus vaccines are not presently available and may be difficult to
216 develop to meet minimum efficacy standards. Recombinant vectored, subunit vaccine candidates that
217 can be produced in scalable vaccine platform expression systems and mRNA-based ASF vaccines are
218 being evaluated in ongoing laboratory research, testing and evaluation in experimental challenge
219 models. The publicly available *Center of Excellence for African Swine Fever Genomics* (ASFV
220 Genomics, 2022¹) that provides the structural protein predictions for all 193 ASFV proteins may help
221 accelerate ASF first and second generation vaccine research and development.

222 Any future use of vaccine candidates should be based on a thorough risk–benefit assessment
223 considering all safety and efficacy features, as well as the potential vaccination scenario. Fit-for-purpose
224 vaccine use scenarios matched to the intended use in a domestic pig-specific type of production system
225 may require different vaccine product profiles or may influence the focus of essential versus ideal
226 vaccine requirements. Prudent use of ASF MLVs as part of strict, controlled vaccination programmes,
227 especially in the areas where ASF is not prevalent, should be implemented.

228 It is important to know what genotypes of ASFV are circulating in a population before vaccination is
229 introduced. Due to the potential risk of recombination events between circulating low and high virulent

¹ <http://asfvgenomics.com>, Accessed 4/4/2023.

230 field strains with future licensed vaccine strains, and the possibility of reversion to virulence of vaccine
231 strains, strict pharmacovigilance post-vaccination is essential. Field pharmacovigilance data should be
232 collected and analysed during vaccination campaigns using ASF MLV first generation vaccines post-
233 licensing. Active post-vaccination surveillance programmes for the detection of new ASF viruses that
234 may arise from MLV vaccine strains and naturally circulating wild-type virus recombination, as well as
235 revertant vaccine strains, should be implemented. It is also recommended that vaccine manufacturers
236 carry out laboratory experiments to further evaluate the risk of vaccine virus recombination with field
237 and vaccine strains.

238 As with any MLV-vaccine, all ASF MLV-vaccines should be used according to the label instructions,
239 under the strict control of the country's Regulatory Authority.

240 The minimum standards given here and in chapter 1.1.8 are intended to be general in nature and may
241 be supplemented by national, regional, and veterinary international medicinal product harmonised
242 requirements. Minimum data requirements for an authorisation in exceptional circumstances (e.g.
243 unexpected introduction of the virus, sudden outbreaks of the disease) should be considered where
244 applicable.

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

246 **2.1. Characteristics of the seed virus**

247 **2.1.1. Biological characteristics of the master seed virus**

248 ASF MLVs are generally produced from ASFV field strains derived from naturally
249 attenuated field isolates or using DNA homologous (genetically targeted) recombination
250 techniques in cell cultures to delete one or more ASFV genes or gene families. These
251 molecular techniques typically involve replacement of the targeted ASFV gene(s) with
252 one or more positive, marker fluorescent (e.g. BFP, eGFP, mCherry) or enzyme-based
253 (e.g. β -glucuronidase) ASFV promoter-reporter gene systems that allow the use of
254 imaging microscopy or flow cytometry to visualise, select, and clone gene-deleted,
255 recombinant, ASF MLVs. MLV production is carried out in cell cultures based on a
256 seed-lot system.

257 Master seed viruses (MSVs) for MLVs should be selected and produced based on their
258 ease of growth in cell culture, virus yield (\log_{10} infectious titre) and genetic stability over
259 multiple cell passages. Preferably, a continuous well-characterised cell line (e.g.
260 ZMAC-4; PIPEC; IPKM) (Borca *et al.*, 2021; Masujin *et al.*, 2021; Portugal *et al.*, 2020)
261 is used to produce a master cell bank (MCB) on which the MSV and MSV-derived
262 working seed virus (WSV) can be produced. The exact source of the underlying ASFV
263 isolate, the whole genome sequence, and the passage history must be recorded.

264 **2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)**

265 Only MSVs that have been established as sterile, pure (free of wild-type parental virus
266 and free of extraneous agents as described in Chapter 1.1.9 *Tests for sterility and*
267 *freedom from contamination of biological materials intended for veterinary use*, and
268 those listed by the appropriate licensing authorities) and immunogenic, should be used
269 as the vaccine virus (WSV and vaccine batch production). Live vaccines must be shown
270 not to cause disease or other adverse effects in target animals in accordance with
271 chapter 1.1.8, Section 7.1 *Safety tests* (for live attenuated MSVs), that includes target
272 animal safety tests, increase in virulence tests, assessing the risk to the environment)
273 and if possible, no transmission to other animals.

274 Identity of the MSV must be confirmed using appropriate methods (e.g. through the use
275 of vaccine strain-specific whole genome detection methods such as next generation
276 sequencing).

277 Demonstration of MSV stability over several cell passages is necessary, typically
278 through at least five passages (e.g. MSV+5). For those MLV vaccines for which
279 attenuation is linked to specific characteristics (gene deletion, gene mutations, etc.),

280 genetic stability of attenuation throughout the production process should be confirmed
281 by full genome sequencing and confirmation of the vaccine phenotype, for example, by
282 confirming the virus titre obtained by growth in the cell line used for production using
283 suitable methods. Suitable techniques to demonstrate genetic stability may include but
284 are not limited to: genome sequencing, biochemical, proteomic, genotypic (e.g.
285 detection of genetic markers) and phenotypic strain characterisation. If final product
286 yields (infectious titres) are relatively low, as is typically the case with ASFV,
287 demonstration of stability is required for the maximum passage for use in the final
288 product manufacturing as defined by the producer genetic stability at a minimum of
289 MSV+10 should be demonstrated to allow more flexibility in the outline of production.
290 For example, if MSV+8 is the maximum passage for use in final product manufacturing,
291 demonstration of genetic stability to at least MSV+10 is warranted.

292 **2.1.3. Validation as a vaccine strain**

293 The vaccine derived from the MSV must be shown to be satisfactory with respect to
294 safety and efficacy.

295 Even if pigs are not known for susceptibility to transmissible spongiform
296 encephalopathy (TSE) agents, consideration should also be given to minimising the
297 risk of TSE transmission by ensuring that animal origin materials from TSE-relevant
298 species, if no alternatives exist for vaccine virus propagation, comply with the measures
299 on minimising the risk of transmission of TSE.

300 Ideally, the vaccine virus in the final product should generally not differ by more than
301 five passages from the master seed lot.

302 ASF vaccines should be presented in a suitable pharmaceutical form (e.g. lyophilisate
303 or liquid form).

304 **2.2. Method of manufacture**

305 **2.2.1. Procedure**

306 The MLV virus is used to infect swine primary cell cultures obtained from specific-
307 pathogen free pigs, the requirements for which are defined in specific monographs
308 (Chapter 2.3.3 *Minimum requirements for the organisation and management of a*
309 *vaccine manufacturing facility*, Section 2.4.2). Compared with primary cell cultures, use
310 of a continuous cell line generally allows for more consistency, higher serial volumes in
311 manufacturing and aligns better with a seed lot system. Thus, preferably a master cell
312 bank based on an established, continuous cell line shown to support genetically stable
313 ASFV replication and acceptable titres over several passages should be used.

314 Cell cultures shall comply with the requirements for cell cultures for production of
315 veterinary vaccines in chapter 1.1.8. Regardless of the production method, the
316 substrate should be harvested under aseptic conditions and may be subjected to
317 appropriate methods to release cell-associated virus (e.g. freeze-thaw cycles,
318 detergent lysis). The harvest can be further processed by filtration and other purification
319 methods. A stabiliser or other excipients may be added as appropriate. The vaccine is
320 homogenised to ensure a uniform batch/serial.

321 **2.2.2. Requirements for ingredients**

322 All ingredients used for vaccine production should be in line with requirements in
323 chapter 1.1.8.

324 **2.2.3. In-process controls**

325 In-process controls will depend on the protocol of production: they include virus titration
326 of bulk antigen and sterility tests.

327 **2.2.4. Final product batch tests**

328 i) Sterility

329 Tests for sterility and freedom from contamination of biological materials intended
330 for veterinary use may be found in chapter 1.1.9.

331 ii) Identity

332 Appropriate methods such as specific genome detection methods (e.g. specific
333 differential real-time PCR) should be used for confirmation of the identity of the
334 vaccine virus and differentiation from the parent strain of the virus as a potential
335 contaminant.

336 iii) Purity

337 Appropriate methods should be used to ensure that the final product batch does
338 not contain any residual wild-type ASFV.

339 iv) Safety

340 Batch safety testing is to be carried out unless consistent safety of the product is
341 demonstrated and approved in the registration dossier and the production process
342 is approved for consistency in accordance with the standard requirements referred
343 to in chapter 1.1.8.

344 v) Batch/serial potency

345 Virus titration is a reliable indicator of vaccine potency once a relationship has
346 been established between the vaccine minimum immunising dose (MID) (minimum
347 protective dose) and titre of the modified live vaccine in vitro. In the absence of a
348 demonstrated correlation between the virus titre and protection, an efficacy test
349 will be necessary (Section C.2.3.3 Efficacy requirements, below).

350 vi) Residual humidity/residual moisture

351 The test should be carried out consistent with VICH² GL26 (*Biologicals: Testing of*
352 *Residual Moisture*, 2003³). Required for MLV vaccines presented as lyophilisates
353 for suspension for injection.

354 **2.3. Requirements for authorisation/registration/licensing**

355 **2.3.1. Manufacturing process**

356 For regulatory approval of a vaccine, all relevant details concerning history of the pre-
357 MSV, preparation of MSV, manufacture of the vaccine and quality control testing
358 (Sections C.2.1 *Characteristics of the seed* and C.2.2 *Method of manufacture*) should
359 be submitted to the authorities.

360 Information shall be provided from three preferably consecutive vaccine batches
361 originating from the same MSV and representative of routine production, with a volume
362 not less than 1/10, and more preferably with a volume not less than 1/3 of the typical
363 industrial batch volume. The in-process controls are part of the manufacturing process.

² VICH: International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medical Products

³ https://www.ema.europa.eu/en/documents/scientific-guideline/vich-gl26-biologicals-testing-residual-moisture-step-7_en.pdf

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2.3.2. Safety requirements

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For the purpose of gaining regulatory approval, the following safety tests should be performed satisfactorily.

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As a minimum standard, vaccines should be tested for any pathogenic effects on healthy domestic pigs of the target age intended for use. Additional demonstration of MLV safety in breeding age gilts and pregnant sows is preferred but not required as a minimum standard. If in the future a vaccine intended for use in breeding animals is developed, an evaluation of the impact of the vaccine on reproductive performance will be a standard safety requirement.

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i) Safety in young animals

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Carry out the test by each recommended route of administration using, in each case, piglets a minimum of 6-4-weeks old and not older than 10-weeks old.

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The test is conducted using no fewer than eight healthy piglets, and preferably no fewer than ten healthy piglets.

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Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

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Administer to each piglet a quantity of the vaccine virus equivalent to not less than ten times the maximum virus titre (e.g. 50% haemadsorption dose [HAD₅₀], 50% tissue culture infective dose [TCID₅₀], quantitative PCR, etc.) (maximum release dose) likely to be contained in one dose of the vaccine.

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To obtain individual and group mean baseline temperatures, the body temperature of each vaccinated piglet is measured on at least the 3 consecutive days preceding administration of the vaccine.

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To confirm the presence or absence of fever accompanied by acute and chronic disease, observe the piglets 4 hours after vaccination and then at least once daily for at least 45 days, preferably 60 days post-vaccination. Carry out the daily observations for signs of acute and chronic disease using a quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.*, 2015a). These clinical signs should include fever, anorexia, recumbency, skin haemorrhage or cyanosis, joint swelling and necrotic lesions around the joints, respiratory distress and digestive findings).

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At a minimum of 45 days post-vaccination, humanely euthanise all vaccinated piglets. Conduct gross pathology on spleen, lung, tonsil, and kidney tissue samples and at least three different lymph nodes (which should include lymph node closest to site of inoculation, gastrohepatic and submandibular nodes).

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The vaccine complies with the test if:

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- No piglet shows abnormal (local or systemic) reactions or notable signs of disease, or reaches the pre-determined humane endpoint defined in the clinical scoring system or dies from causes attributable to the vaccine;

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- The average body temperature increase for all vaccinated piglets (group mean) for the observation period does not exceed 1.5°C above baseline; and no individual piglet shows a temperature rise above baseline greater than 2.5°C for a period exceeding 3 days.

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- On each day during the observation period, the maximum increase in body temperature above the baseline observed for each pig will be used to calculate the daily group mean temperature rise. This mean value should not

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410 exceed 1.5°C and no individual pig should show a rise in temperature above
411 baseline greater than 1.5°C for a period exceeding 3 consecutive days.

412 • No vaccinated pigs show notable signs of disease by gross pathology

413 ii) Safety test in pregnant sows and test for transplacental transmission

414 There is limited currently an absence of published information on ASFV
415 pathogenesis in breeding-age gilts and in pregnant sows associated with ASFV
416 transplacental infection and fetus abortion/stillbirth. If a label claim is pursued for
417 use in breeding age gilts and sows, then a safety study in line with VICH GL44
418 (Guidelines on Target Animal Safety for Veterinary Live and Inactivated Vaccines,
419 Section 2.2. Reproductive Safety Test, 2009⁴) should be completed.

420 iii) Horizontal transmission

421 The test is conducted using no fewer than 12 healthy piglets, a minimum of 6-4-
422 weeks old and not older than 10-weeks old and of the same origin, that do not
423 have antibodies against ASFV, and blood samples are negative on real-time PCR.
424 All piglets are housed together from day 0 and the number of vaccinated animals
425 is the same as the number of naïve, contact animals. Co-mingle equal numbers of
426 vaccinated and naïve, contact piglets from day 0 in the same pen or room.

427 Use vaccine virus at the least attenuated passage level that will be present
428 between the master seed lot and a batch of the vaccine. Administer by each
429 recommended route of administration to no fewer than six piglets a quantity of the
430 vaccine virus equivalent to not less than the maximum virus titre (maximum
431 release dose) likely to be contained in 1 dose of the vaccine.

432 To obtain individual and group mean baseline temperatures, the body temperature
433 of each naïve, contact piglet is measured on at least the 3 consecutive days
434 preceding co-mingling with vaccinated piglets. The body temperature of each
435 naïve, contact piglet is then measured daily for at least 45 days, preferably 60
436 days.

437 To confirm the presence or absence of fever accompanied by disease, observe
438 the naïve, contact piglets daily for at least 45 days, preferably 60 days. On each
439 day during the observation period the maximum increase in body temperature
440 above the baseline observed for each pig will be used to calculate the daily group
441 mean temperature rise. This mean value should not exceed 1.5°C and no
442 individual pig should show a rise in temperature above baseline greater than 1.5°C
443 for a period exceeding 3 consecutive days. Carry out the daily observations for
444 signs of acute and chronic clinical disease using a quantitative clinical scoring
445 system adding the values for multiple clinical signs (e.g. Gallardo *et al.*, 2015a).
446 These clinical signs should include fever, anorexia, recumbency, skin
447 haemorrhage or cyanosis, joint swelling and necrotic lesions around the joints,
448 respiratory distress and digestive findings.

449 In addition, Blood should be taken from the naïve contact piglets at least twice a
450 week for the first 21 days post-vaccination and then on a weekly basis. From the
451 blood samples, determine vaccine virus titres by quantitative virus isolation
452 (HAD₅₀/ml, TCID₅₀/ml or other methods, e.g. titration using IPT or FAT detection).
453 Quantitative PCR may be used to detect positive samples, but results should be
454 confirmed by infectious virus titration as described above infectious virus titres by
455 quantitative virus isolation (e.g. HAD₅₀/ml or TCID₅₀/ml) and using a real-time PCR
456 test.

⁴ https://www.ema.europa.eu/en/documents/scientific-guideline/vich-gl44-target-animal-safety-veterinary-live-inactivated-vaccines-step-7_en.pdf

457 If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a
458 real-time PCR test only may be used.

459 Collect blood (serum) samples from the naïve contact pigs at least at day 21 and
460 day 28 days and carry out an appropriate test to detect vaccine virus antibodies.
461 At a minimum of 45 days, humanely euthanise all naïve, contact piglets. Conduct
462 gross pathology on spleen, lung, tonsil, and kidney tissue samples and at least
463 three different lymph nodes. Determine virus titres in all collected samples by
464 quantitative virus isolation (HAD₅₀/mg or TCID₅₀/mg) or other appropriate methods
465 (e.g. IPT or FAT detection). Quantitative PCR may be used to detect positive
466 samples, but results should be confirmed by infectious virus titration as described
467 above and real-time (RT)-PCR (see Section B.1. Identification of the agent). If the
468 vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-
469 time PCR test or other appropriate method (e.g. titration using IPT or FAT
470 detection) may be used.

471 The vaccine complies with the test if:

472 • No vaccinated or naïve contact piglet shows abnormal (local or systemic)
473 reactions or notable signs of disease, reaches the predetermined humane
474 endpoint defined in the clinical scoring system or dies from causes
475 attributable to the vaccine;

476 • On each day during the observation period the maximum increase in body
477 temperature above the baseline observed for each pig will be used to
478 calculate the daily group mean temperature rise. This mean value should not
479 exceed 1.5°C and no individual pig should show a rise in temperature above
480 baseline greater than 1.5°C for a period exceeding 3 consecutive days. The
481 average body temperature increase for all naïve, contact piglets (group mean)
482 for the observation period does not exceed 1.5°C above baseline; and no
483 individual piglet shows a temperature rise above baseline greater than 2.5°C
484 for a period exceeding 3 days;

485 • No naïve, contact piglet shows notable signs of disease by gross pathology
486 and no virus is detected in their blood or tissue samples;

487 • No or a low percentage of contact piglets test both real-time PCR positive and
488 seropositive. No naïve contact pigs test positive for antibodies to the vaccine
489 virus.

490 iv) Post-vaccination kinetics of viral replication (MLV blood and tissue dissemination)
491 study

492 Prior to the reversion to virulence study (Section C2.3.2.v. below), a minimum of
493 one study should be performed to determine the post-vaccination kinetics of virus
494 replication in the blood (viremia), tissues and viral shedding.

495 The test consists of the administration of the vaccine virus from the master seed
496 lot to no fewer than eight healthy piglets, and preferably ten healthy piglets, a
497 minimum of 6-4-weeks old and not older than 10-weeks old and of the same origin,
498 that do not have antibodies against ASFV, and blood samples are negative on
499 real-time PCR.

500 Administer to each piglet, using the recommended route of administration most
501 likely to result in spread (such as the intramuscular route or intranasal route), a
502 quantity of the master seed vaccine virus equivalent to not less than the maximum
503 virus titre (maximum release dose) likely to be contained in 1 dose of the final
504 product of the vaccine.

505 Record daily body temperatures and observe inoculated animals daily for clinical
506 disease for at least 45 days, preferably 60 days.

507 Carry out the daily observations for signs of acute and chronic clinical disease
508 using a quantitative clinical scoring system adding the values for multiple clinical
509 signs (e.g. Gallardo *et al.* (2015a). These clinical signs should include fever,
510 anorexia, recumbency, skin haemorrhage or cyanosis, joint swelling and necrotic
511 lesions around the joints, respiratory distress and digestive findings.

512 Collect blood samples from all the piglets at least two times per week from 3 days
513 post-vaccination for the first 2 weeks, then weekly for the duration of the test.
514 Determine vaccine virus titres by quantitative virus isolation (HAD₅₀/ml or
515 TCID₅₀/ml) or other appropriate methods (e.g. titration using IPT or FAT detection).
516 Quantitative PCR may be used to detect positive samples but results should be
517 confirmed by infectious virus titration as described above and using a real-time
518 PCR test. If the vaccine virus is non-haemadsorbing or does not cause cytopathic
519 effects, a real-time PCR test only may be used.

520 Determine which blood timepoint(s) should be used in the design of the reversion
521 to virulence study (Section C2.3.2.v. below), for example, specific blood sample(s)
522 at specific timepoints that show the highest titres should be considered for
523 selection and use in the reversion to virulence study.

524 Collect oral, nasal and faecal swab samples (preferably devoid of blood to
525 minimise assay interference) at least two times per week from 3-days post-
526 vaccination for the first 2 weeks, then weekly for the duration of the test. Test the
527 swabs for the presence of vaccine virus. Determine virus titres in all collected
528 samples by quantitative virus isolation (HAD₅₀/ml or TCID₅₀/ml) or other
529 appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR
530 may be used to detect positive samples, but results should be confirmed by
531 infectious virus titration as described above and using a real-time PCR test. If the
532 vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-
533 time PCR test or other appropriate method (e.g. titration using IPT or FAT
534 detection) may be used.

535 Euthanise at least two piglets on days 5, 7, 14, 21, and preferably on day 28 (±2
536 days at each timepoint) and collect spleen, lung, tonsil, kidney tissue samples and
537 at least three different lymph nodes (which should include lymph node closest to
538 site of inoculation, gastrohepatic and submandibular nodes). Determine virus titres
539 in all collected samples by quantitative virus isolation (HAD₅₀/mg or TCID₅₀/mg) or
540 other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative
541 PCR may be used to detect positive samples, but results should be confirmed by
542 infectious virus titration as described above and using real-time PCR test. If the
543 vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-
544 time PCR test or other appropriate method (e.g. titration using IPT or FAT
545 detection) may be used.

546 Determine which tissue(s) and timepoint(s) should be used to aid in the design of
547 the reversion to virulence study (Section C.2.3.2.v), for example, specific tissues
548 at specific timepoints which show the highest titres should be considered for
549 selection and use in the reversion to virulence study.

550 v) Reversion to virulence

551 The test carried out should be consistent with VICH GL41 (Examination of live
552 veterinary vaccines in target animals for absence of reversion to virulence, 2008⁵).

553 The test for increase in virulence consists of the administration of the vaccine
554 master seed virus to healthy piglets of an age (e.g. between 6-4 weeks and 10
555 weeks old) suitable for recovery of the strain and of the same origin, that do not

⁵ https://www.ema.europa.eu/en/documents/scientific-guideline/vich-gl41-target-animal-safety-examination-live-veterinary-vaccines-target-animals-absence-reversion_en.pdf

556 have antibodies against ASFV, and blood samples that are negative on real-time
557 PCR. This protocol is typically repeated five times.

558 First passage (p1)

559 Administer to no fewer than two piglets, and preferably no fewer than four piglets
560 using the intended route of administration for the final product, a quantity of the
561 master seed vaccine virus equivalent to not less than the maximum virus titre
562 (maximum release dose) likely to be contained in 1 dose of the final product of the
563 vaccine. Observe inoculated animals daily for the appearance of at least two and
564 preferably at least three clinical signs and record daily body temperatures using a
565 quantitative clinical scoring system adding the values for multiple clinical signs
566 (e.g. Gallardo *et al.*, 2015a) and record daily body temperatures.

567 Based on results from at least one completed post-vaccination kinetics of viral
568 replication (MLV vaccine shed and spread (virus blood and tissue dissemination
569 study (Section C.2.3.2.iv above), collect an appropriate quantity of blood from each
570 piglet on the predetermined single timepoint(s) (day 5–3–13). Determine virus titres
571 in individual blood-samples by quantitative virus isolation (HAD₅₀/ml or TCID₅₀/ml)
572 or other appropriate methods (e.g. titration using IPT or FAT detection).
573 Quantitative PCR may be used to detect positive samples, but results should be
574 confirmed by infectious virus titration as described above and by real-time PCR. If
575 the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a
576 real-time PCR test or other appropriate method (e.g. titration using IPT or FAT
577 detection) may be used. Identify the individual blood sample(s) with the highest
578 infectious titre and reserve for the subsequent *in-vivo* passage (second pass, p2).

579 Based on results from at least one completed vaccine virus-MLV blood and tissue
580 distribution-dissemination study (Section C.2.3.2.iv above), euthanise piglets on
581 the predetermined timepoint (i.e. day 5, 7, 14, 21, or 28). Determine infectious
582 virus titres in individual tissue samples by quantitative virus isolation (HAD₅₀/ml or
583 TCID₅₀/ml) or other appropriate methods (e.g. titration using IPT or FAT detection).
584 Quantitative PCR may be used to detect positive samples, but results should be
585 confirmed by infectious virus titration as described above. If the vaccine virus is
586 non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test or
587 other appropriate method (e.g. titration using IPT or FAT detection) may be used.
588 Identify individual tissue-sample type(s) with the highest infectious titre. Pool the
589 tissues with the highest titres from different organs from all each animals with the
590 highest titres and prepare at least a 10% virus suspension to obtain a virus titre
591 within the range used for inoculation in PBS, pH 7.2 kept at 4°C or at -70°C for
592 longer storage.

593 Test each blood and tissue sample pool used for inoculation by PCR to confirm
594 the absence of potential viral agent contaminants (i.e. CSFV, FMDV, PRRS,
595 PCV2). Blood and pooled tissue (p1) are used to inoculate 2 ml of positive material
596 diluted to the maximum release dose likely to be contained in 1 dose of the vaccine
597 using the intended route of administration for the final product to each of at least
598 two and ideally at least four further pigs of the same age and origin.

599 Second pass (p2)

600 If no virus is found at passage 1 (p1), repeat the administration by the intended
601 route once again with the same pooled material (blood and pooled tissue, p1) in
602 another ten healthy piglets of the same age and origin. If no virus is found at this
603 point during this second passage (p2) at this point, end the process here.

604

Second passage (p2)

605 If ~~however~~ virus is found in p1, carry out a second series of passages by
606 administering 2 ml of positive material diluted to the maximum release dose likely
607 to be contained in 1 dose of the vaccine using the intended route of administration
608 for the final product to each of no fewer than two piglets, and preferably no fewer
609 than four piglets of the same age and origin. Observe inoculated animals daily for
610 the appearance of at least two and preferably at least three clinical signs using a
611 quantitative clinical scoring system adding the values for multiple clinical signs
612 (e.g. Gallardo *et al.*, 2015a), and record daily body temperatures and determine
613 infectious virus titres in individual blood and tissue samples as described for p1
614 above.

615

Third and fourth pass (p3 and p4)

616 If no virus is found at in (p2), repeat the intramuscular administration by the
617 intended route once again with the same pooled material (blood and pooled tissue,
618 p2) in another eight healthy piglets of the same age and origin. If no virus is found
619 at this point, end the process here.

620

Third and fourth passage (p3 and p4)

621 If, ~~however~~, virus is found on p2, carry out this passage operation no fewer than
622 two additional times (p3 and p4) (to each of no fewer than two piglets, and
623 preferably no fewer than four piglets of the same age and origin) and verifying the
624 presence of the virus at each passage in blood and tissues. Observe inoculated
625 animals daily for the appearance of at least two and preferably at least three
626 clinical signs using a quantitative clinical scoring system adding the values for
627 multiple clinical signs (e.g. Gallardo *et al.*, 2015a) and record daily body
628 temperatures.

629

Fifth passage (p5)

630 Administer 2 ml of the blood and pooled tissue (p4) to each of at least eight healthy
631 piglets of the same age and origin. Observe inoculated animals daily for at least
632 28 days post-inoculation for the appearance of at least two and preferably at least
633 three clinical signs using a quantitative clinical scoring system adding the values
634 for multiple clinical signs (e.g. Gallardo *et al.*, 2015a), and record daily body
635 temperature and determine infectious virus titres in individual blood and tissue
636 samples as described above.

637

The vaccine virus complies with the test if:

- 638 • No piglet shows abnormal (local or systemic) reactions, or notable signs of
639 disease, or reaches the pre-determined humane endpoint defined in the
640 clinical scoring system or dies from causes attributable to the vaccine; and
- 641 • There is no indication of increasing virulence (as monitored by daily body
642 temperature accompanied by clinical sign observations) of the maximally
643 passed virus compared with the master seed virus.

644

At a minimum, a safe MLV vaccine shall demonstrate ALL the following features
645 (minimal standards):

- 646 • Absence of fever (on each day during the observation period, the maximum
647 increase in body temperature above the baseline observed for each pig will be
648 used to calculate the daily group mean temperature rise. This mean value
649 should not exceed 1.5°C and no individual pig should show a rise in
650 temperature above baseline greater than 1.5°C (defined as average body
651 temperature increase for all vaccinated piglets (group mean) for the
652 observation period does not exceed 1.5°C above baseline; and no individual

653 piglet shows a temperature rise above baseline greater than 2.5°C for a period
654 exceeding 3 days);

655 • Absence of chronic and acute clinical signs and gross pathology over the
656 entire test period or minimal chronic mild clinical signs (defined as e.g. mild
657 swollen joints with a low clinical score that resolve within 1 week).

658 • Minimal (defined as no naïve, contact piglet shows notable signs of disease
659 by clinical signs and gross pathology and no or a low percentage of contact
660 piglets test both real-time PCR positive and seropositive) or no vaccine virus
661 transmission (defined as no naïve, contact piglet shows notable signs of
662 disease by clinical signs and gross pathology and no contact piglets test both
663 real-time PCR positive and seropositive) over the entire test period;

664 • Absence of an increase in virulence (genetic and phenotypic stability)
665 (complies with the reversion to virulence test).

666 In addition, for regulatory approval, ASF MLV the vaccines in their commercial
667 presentation before being authorised for general use should be tested for safety
668 in the under field conditions (see chapter 1.1.8 Section 7.2.3). Additional Field
669 safety studies generally evaluation studies may include measurement of body
670 temperatures, observation of local or systemic reactions and, where appropriate,
671 performance measurements but are not limited to: environmental persistence (e.g.
672 determination of virus recovery from bedding or other surfaces), assessment of
673 immunosuppression, and negative impacts on performance.

674 **2.3.3. Efficacy requirements**

675 i) Protective dose

676 Vaccine efficacy is estimated in immunised animals directly, by evaluating their
677 resistance to live virus challenge. The test consists of a vaccination/challenge trial
678 in piglets a minimum of 6-4-weeks old and not more than 10-weeks old, free of
679 antibodies to ASFV, and negative blood samples by real-time PCR. The test is
680 conducted using no fewer than 15 and preferably no fewer than 24 vaccinated
681 pigs, and no fewer than five non-vaccinated control piglets.

682 The test is conducted to determine the minimal immunising dose (MID) (also
683 referred to as the minimal protective dose [MPD] or protective fraction); using at
684 least three groups of no fewer than five and preferably not fewer than eight
685 vaccinated piglets per group, and one additional group of no fewer than five non-
686 vaccinated piglets of the same age and origin as controls. Use vaccine containing
687 virus at the highest passage level that will be present in a batch of vaccine.

688 Each group of piglets, except the control group, is immunised with a different
689 vaccine virus content in the same vaccine volume. In at least one vaccinated
690 group, piglets are immunised with a vaccine dose containing not more than the
691 minimum virus titre (minimum release dose) likely to be contained in one dose of
692 the vaccine as stated on the label.

693 Twenty-eight days (±2 days) after the single injection-dose of vaccine (or if using
694 two injections doses of the vaccine then 28 days [±2 days] following the second
695 injection-dose), challenge all the piglets by the intramuscular route. If previous
696 studies have demonstrated acceptable efficacy using IM challenge, then a
697 different challenge route (e.g. direct contact, oral or oronasal) may be used.
698 Challenged, vaccinated piglets may be housed in one or more separate pens in
699 the same room or in different rooms. Challenged, naïve controls can be housed in
700 one or more rooms that are separate from challenged, vaccinated piglets.

701 Carry out the test using an ASFV representative strain of the epidemiologically
702 relevant field strain(s) where the vaccine is intended for use (e.g. ASFV B646L
703 [p72] genotype II pandemic strain and other p72 virulent genotype of recognised

704 epidemiologic importance). For gene deleted, recombinant MLV viruses, if neither
705 challenge virus type is available, then carry out the test with the parental, virulent
706 virus used to generate the MLV recombinant virus. Use a 10e3–10e4 HAD₅₀ (or
707 TCID₅₀ for non-HAD viruses) challenge dose sufficient to cause death or meet the
708 humane endpoint in 100% of the nonvaccinated piglets in less than 21 days.
709 Higher or lower challenge doses can be considered if appropriately justified.

710 The rectal temperature of each vaccinated piglet is measured on at least the 3
711 days preceding administration of the challenge virus, at the time of challenge, 4
712 hours after challenge, and then daily for the observation period of at least 28–45
713 days, preferably 35–60 days. Observe the piglets at least daily for at least 28 days,
714 preferably 35 days. Carry out the daily observations for signs of acute and chronic
715 clinical disease using a quantitative clinical scoring system adding the values for
716 multiple clinical signs (e.g. Gallardo *et al.*, 2015). These clinical signs should
717 include fever, anorexia, recumbency, skin haemorrhage or cyanosis, joint swelling
718 and necrotic lesions around the joints, respiratory distress and digestive findings.

719 Collect oral, nasal, anal and blood samples from the vaccinated challenged piglets
720 at least two times ~~once~~ per week from 3 days post-challenge for at least 28–14
721 days, then weekly up to 35 days post-challenge and then every 14 days up to the
722 end of the observation period preferably 35 days. From the blood samples,
723 determine infectious virus titres by quantitative virus isolation (HAD₅₀/ml or
724 TCID₅₀/ml) or other appropriate methods (e.g. titration using IPT or FAT detection).
725 Quantitative PCR may be used to detect positive samples, but results should be
726 confirmed by infectious virus titration as described above and using a real-time
727 PCR test. If the vaccine virus is non-haemadsorbing or does not cause cytopathic
728 effects, a real-time PCR test only may be used.

729 At the end of the test period, humanely euthanise all vaccinated challenged piglets.
730 Conduct gross pathology (and histopathology if considered necessary) on spleen,
731 lung, tonsil, and kidney tissue samples and at least three different lymph nodes
732 (which should include lymph node closest to site of inoculation, gastrohepatic and
733 submandibular nodes). Determine virus titres in all collected samples by
734 quantitative virus isolation (HAD₅₀/mg or TCID₅₀/mg) or other appropriate methods
735 (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect
736 positive samples, but results should be confirmed by infectious virus titration as
737 described above and real-time PCR (see Section B.1. Identification of the agent).
738 If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a
739 real-time PCR test or other appropriate method (e.g. titration using IPT or FAT
740 detection) may be used.

741 The test is invalid if fewer than 100% the difference between in the number of
742 unvaccinated control piglets infected with the live challenge virus and the number
743 of vaccinated / challenged piglets vaccinated with the minimum release dose that
744 die or reach a humane endpoint is not statistically significant.

745 The vaccine (or a specific vaccine virus dose if conducting a vaccine dose titration
746 study) complies with the test if:

- 747 • No vaccinated challenged piglet dies or shows abnormal (local or systemic)
748 reactions, reaches the humane endpoint or dies from causes attributable to
749 ASF;
- 750 • On each day during the observation period the maximum increase in body
751 temperature above the baseline observed for each pig will be used to
752 calculate the daily group mean. This mean value should not exceed 1.5°C
753 and no individual pig should show a rise in temperature above baseline
754 greater than 2.0°C for a period exceeding 2 consecutive days. The average
755 body temperature increase for all vaccinated challenged piglets (group mean)
756 for the observation period does not exceed 2.0°C above baseline; and no
757 individual piglet shows a temperature rise above baseline greater than 2.0°C;

758 • The vaccinated challenged piglets display a reduction or absence of typical
759 acute clinical signs of disease and gross pathology and a reduction or
760 absence of challenge virus levels in blood and tissues.

761 ii) Assessment for horizontal transmission (challenge virus shed and spread study)

762 The ASF basic reproduction number, R₀, can be defined as the average number
763 of secondary ASF disease cases caused by a single ASFV infectious pig during
764 its entire infectious period in a fully susceptible population (Hayes *et al.*, 2021). In
765 general, if the ASFV effective reproduction number $Re=R_0 \times (S/N)$ (S= susceptible
766 pigs; N= total number of pigs in a given population) is greater than 1.0, disease is
767 predicted to spread. Ideally, ASF vaccination should reduce Re to less than 1.0 by
768 reducing the number of susceptible, naïve, contact pigs exposed to vaccinated,
769 infected pigs.

770 To evaluate ASF vaccine impact on ASF disease transmission, the test consists
771 of a vaccination/challenge trial in piglets a minimum of 6-4-weeks old and not older
772 than 10-weeks old, free of antibodies to ASFV, and negative blood samples by
773 real-time PCR.

774 The test is conducted using no fewer than 15 healthy piglets at a ratio comprising
775 twice the number of vaccinated piglets to naïve piglets (e.g. ten vaccinated and
776 five naïve). Use vaccine containing virus at the highest passage level that will be
777 present in a batch of the vaccine.

778 The quantity of vaccine virus administered to each pig is equivalent to be not less
779 than the minimum virus titre (minimum dose) likely to be contained in one dose of
780 the vaccine as stated on the label. Following immunisation, vaccinated and naïve
781 piglets should continue to be co-mingled.

782 Twenty-eight days (± 2 days) after the single injection-dose of vaccine (or if using
783 two injections doses of the vaccine then 28 days [± 2 days] following the second
784 injection-dose), temporarily separate [into different pen(s) or room(s)] all
785 vaccinated piglets from naïve piglets. Challenge all vaccinated piglets by the
786 intramuscular or other previously verified route. Carry out the challenge using an
787 ASFV representative strain of the epidemiologically relevant field strain(s) where
788 the vaccine is intended for use (e.g. ASFV B646L [p72] genotype II pandemic
789 strain and other p72 virulent genotype of recognised epidemiological importance).
790 For gene deleted, recombinant MLV viruses, if neither challenge virus type is
791 available, then carry out the test with the parental, virulent virus used to generate
792 the MLV recombinant virus. Use a $10e3-10e4$ HAD₅₀ (or TCID₅₀ for non-HAD
793 viruses challenge dose sufficient to cause death or met the humane endpoint in
794 100% of the nonvaccinated piglets in less than 21 days. Higher or lower challenge
795 doses can be considered if appropriately justified.

796 Approximately 18–24 hours later, re-introduce naïve piglets to vaccinated,
797 challenged piglets and allow for direct nose to nose contact exposure with
798 vaccinated, challenged piglets. Allow for continuous contact exposure by co-
799 mingling both groups through the end of the study. If more than one pen or room
800 is used for co-housing, following reintroduction initially maintain a ratio of 2:1 of
801 challenged, vaccinated piglets to contact exposed, naïve piglets.

802 The rectal temperature of each contact piglet is measured on at least the 3 days
803 preceding administration of the challenge virus to vaccinated pigs, immediately
804 prior to direct contact exposure, 4 hours post-contact exposure, and then daily for
805 at least 28, preferably 35 days and twice a week for at least 60 days. Observe all
806 contact exposed piglets at least daily for at least 28 days, and then twice a week
807 for at least 60 days preferably for at least 35 days.

808 Carry out the daily observations in each contact piglet for signs of acute and
809 chronic clinical disease using a quantitative clinical scoring system adding the
810 values for multiple clinical signs (e.g. Gallardo *et al.*, 2015a). These clinical signs
811 should include fever, anorexia, recumbency, skin haemorrhage or cyanosis, joint
812 swelling and necrotic lesions around the joints, respiratory distress and digestive
813 findings.

814 In addition, blood should be taken from the naïve contact piglets at least twice a
815 week from 3 days post-contact exposure for the duration collect blood samples
816 from the contact piglets at least two times per week from 3 days post-contact for
817 at least 14 days, then weekly up to 35 days post-contact exposure and then every
818 14 days up to the end of the test period. Determine virus titres in all collected
819 samples by quantitative virus isolation (HAD₅₀/mg or TCID₅₀/mg) or other
820 appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR
821 may be used to detect positive samples, but results should be confirmed by
822 infectious virus titration as described above. From the blood samples, determine
823 infectious challenge virus titres by quantitative virus isolation (HAD₅₀/ml or
824 TCID₅₀/ml) and using a real-time PCR test. If the vaccine virus is non-
825 haemadsorbing or does not cause cytopathic effects, a real-time PCR test only
826 may be used.

827 Collect blood (serum) samples from the naïve contact pigs at least at day 21 and
828 day 28 (±2 days), and at the end of the test period, and carry out an appropriate
829 test to detect vaccine virus antibodies.

830 Collect oral, nasal and faecal swab samples (preferably devoid of blood to
831 minimise assay interference) from all contact-exposed naïve piglets at least two
832 times per week from 3-days post-contact exposure for the first 2 weeks, then
833 weekly for the duration of the test and test swabs for the presence of challenge
834 virus. Determine virus titres in all collected samples by quantitative virus isolation
835 (HAD₅₀/mg or TCID₅₀/mg) or other appropriate methods (e.g. titration using IPT or
836 FAT detection). Quantitative PCR may be used to detect positive samples, but
837 results should be confirmed by infectious virus titration as described above
838 Determine virus titres in all collected samples by quantitative virus isolation
839 (HAD₅₀/ml or TCID₅₀/ml) and using a real-time PCR test. If the vaccine virus is non-
840 haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other
841 appropriate method (e.g. titration using IPT or FAT detection) may be used.

842 At the end of the test period, humanely euthanise all contact piglets. Conduct gross
843 pathology on spleen, lung, tonsil, and kidney tissue samples and at least three
844 different lymph nodes. (which should include lymph node closest to site of
845 inoculation, gastrohepatic and submandibular nodes). Determine virus titres in all
846 collected samples by quantitative virus isolation (HAD₅₀/mg or TCID₅₀/mg) or other
847 appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR
848 may be used to detect positive samples, but results should be confirmed by
849 infectious virus titration as described above. Determine virus titres in all collected
850 samples by quantitative virus isolation (HAD₅₀/mg or TCID₅₀/mg) and real-time
851 PCR (see Section B.1. Identification of the agent). If the vaccine virus is non-
852 haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other
853 appropriate method (e.g. titration using IPT or FAT detection) may be used.

854 The test is invalid if the vaccine fails to comply with the compliance criteria
855 described for the protected dose test in vaccinated pigs (Section C.2.3.3.i above).

856 If the manufacturer claims that the vaccine induces sterilising immunity, the
857 vaccine complies with the test for a reduction in horizontal disease transmission if
858 all the following conditions are satisfied:

- 859 • No naïve, contact exposed piglet shows abnormal (local or systemic)
860 reactions, reaches the defined humane endpoint or dies from causes
861 attributable to ASF;

862 • No naïve, contact exposed piglet displays fever accompanied by typical signs
863 of disease, including gross pathology.

864 • Naïve contact pigs show an absence of challenge virus in blood and tissues.

865 • No naïve contact pigs test positive for antibodies to the challenge virus.

866 Otherwise, the vaccine complies with the test for a reduction in horizontal disease
867 transmission if:

868 • Naïve contact pigs show a reduction or absence of challenge virus levels in
869 blood and tissues.

870 • None of or a reduced number of naïve contact exposed pigs test positive for
871 antibodies to the challenge virus.

872 At a minimum, an efficacious MLV vaccine shall demonstrate ALL the following
873 features (minimal standards):

874 • Protects against mortality;

875 • Reduces acute disease (fever accompanied by a reduction of typical clinical
876 and pathological signs of acute disease)

877 • Reduces levels of viral shedding and viraemia.

878 • Reduces horizontal disease transmission (~~no~~ none of or a reduced number
879 of naïve, contact exposed piglets shows abnormal [local or systemic]
880 reactions, reaches the humane endpoint or dies from causes attributable to
881 ASF, and displays fever accompanied by typical acute disease signs caused
882 by ASF) and test positive for antibodies to the challenge virus.

883 • Reduces levels of viral shedding and viraemia.

884 In general, for regulatory approval, ASF MLV addition, the vaccines in their commercial
885 presentation before being authorised for general use should be tested for efficacy in
886 the under field conditions (see chapter 1.1.8 Section 7.2.3). Additional Field efficacy
887 evaluation studies may generally include but are not limited to: onset of immunity,
888 duration of immunity, and impact on disease transmission measurement of relevant
889 efficacy parameters including but limited to mortality, clinical signs, impact on disease
890 transmission, performance parameters.

891 **2.3.4. Duration of immunity**

892 Although not included in the guidance for ASF MLV first generation vaccines,
893 manufacturers are ~~encouraged~~ required, as part of the authorisation procedure, to
894 define and demonstrate the duration of immunity of a given vaccine by evaluation of
895 potency at the end of the claimed period of protection.

896 **2.3.5. Stability**

897 Stability of the vaccine should be demonstrated over the shelf life recommended for the
898 product. Although not included in the standards for first generation MLV ASF vaccines,
899 manufacturers are ~~encouraged~~ required, as part of the authorisation procedure, to
900 generate data supporting the retention of immunogenicity over a defined period of
901 validity time of a lyophilised or other pharmaceutical form of the ASF vaccine as part of
902 the authorisation procedure.

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1095 **NB:** There are WOA Reference Laboratories for African swine fever
1096 (please consult the WOA Web site:
1097 <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>).
1098 Please contact the WOA Reference Laboratories for any further information on
1099 diagnostic tests and reagents for African swine fever

1100 **NB:** FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2021.