# Report of the Meeting of the WOAH Biological Standards Commission

Original: English (EN)

5 to 9 February 2024

Paris

**Introduction and Member contribution**

This report presents the work of the WOAH Biological Standards Commission (hereinafter called 'the Commission') who met in Paris, France from 5 to 9 February 2024.

During the meeting, 13 chapters from the WOAH *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (*Terrestrial Manual*) were approved for circulation for second-round Member comment and proposal for adoption at the General Session in May 2024. The Commission wished to thank the following Members for providing comments on draft texts for the WOAH *Terrestrial Manual* circulated with the Commission's September 2023 report: Canada, China (People's Rep. of), Japan, New Zealand, Switzerland, the United Kingdom (UK), the United States of America (USA), and the Member States of the European Union (EU). The Commission also wished to acknowledge the valuable advice and contributions from numerous experts of the WOAH scientific network.

The Commission reviewed all comments that were submitted prior to the deadline and were supported by a rationale. Where amendments were of an editorial nature, no explanatory text has been provided. The Commission wished to note that when texts proposed by Members to improve clarity were not accepted, it considered the text was clear as currently written. The Commission made amendments to draft texts, where relevant, in the usual manner by 'double underline' and 'strikethrough'. In relevant annexes, amendments proposed at this meeting are highlighted in yellow to distinguish them from those made previously.

Your participation in the WOAH standard-setting process is valued. Thank you for your engagement in the process!

During the meeting, ten Reference Centre applications and ten nominations for replacement experts were also evaluated.

**Annexes**

Texts in Annexes 4 to 16 will be proposed for adoption at the 91st General Session in May 2024.

**How to submit comments**

The Biological Standards Commission strongly encourages WOAH Members and International Organisations with a WOAH Cooperation Agreement to participate in the development of WOAH International Standards by submitting comments on relevant annexes of this report.

Engagement of Members and International Organisations in the standard-setting process through the submission of comments is critical to ensure the Commission's work is science based and takes into consideration the different contexts among Members and stakeholders, and enables the implementation of standards. To ensure that comments are considered they should be submitted by the deadline and in the format described in the guidance and SOP documents available on the Delegate's website and the WOAH public website.

Comments that are not correctly formatted as described in the guidance, may not be considered by the Commission. Any questions on the requirements for formatting and submission of comments should be sent to BSC.Secretariat@woah.org

The Biological Standards Commission wished to highlight that when a Commission discussion is based on the input of an *ad hoc* Group, Members are encouraged to review the relevant *ad hoc* Group report together with the report of the Commission. *Ad hoc* Group reports are available on the dedicated webpages on the WOAH website at Ad hoc Groups - WOAH - World Organisation for Animal Health.

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**Deadline to comment**

Comments on relevant texts in this report must reach the Headquarters by 30 April 2024 to be considered by the Biological Standards Commission.

**Where to send comments**

All comments should be sent to the Science Department at: BSC.Secretariat@woah.org

**Date of the next meeting**

The Biological Standards Commission noted the dates for its next meeting will be confirmed following the Commission election at the 91st General Session in May 2024.

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## 1. Welcome from the Directors

### 1.1. Director General

Dr Monique Eloit, the WOAH Director General, met the Biological Standards Commission on 6 February and thanked its members for their support and commitment to achieving WOAH objectives.

Dr Eloit underlined that this meeting marked the conclusion of the current term of the Commission and expressed her gratitude to the members for their consistent efforts throughout their years of collaboration. With the term drawing to a close, a call for applications for members was issued last August. The list of candidates will be presented to the Council at their March meeting, followed by discussions and negotiations among the regions. The election for the four WOAH Commissions is scheduled to take place during the forthcoming General Session.

Dr Eloit informed the Commission about WOAH's ongoing consultancy project aimed at evaluating the organisation's *Basic Texts* from both a technical and legal perspective. This revision seeks to enhance WOAH's internal systems, reinforce its credibility, and strengthen its global standing. The consultancy focuses on three main pillars: institutional matters; the science system, which encompasses the ToR[1] for both the Commissions and Reference Centres; and the organisation's business model. The goal of analysing the *Basic Texts* is to facilitate a comprehensive review and to present the findings to the Assembly. Dr Eloit also noted that selected members from the four Commissions will play a significant role in the revision process of the *Basic Texts*.

In her closing remarks, Dr Eloit provided an update on the progress being made on the Pandemic Treaty with the WHO[2]. She highlighted that this treaty will formally recognise the importance of disease prevention, including animal health. Additionally, there will be an increased focus on research within the animal sector, emphasising the crucial role of vaccines. Dr Eloit also stressed the need to not only promote the use of existing vaccines but also to invest significantly in the development of new vaccines. This approach underscores a proactive strategy in disease management and prevention, particularly in the animal sector, aligning with the broader goals of global health and safety.

The Commission thanked Dr Eloit for these updates.

### 1.2. Deputy Director General, International Standards and Science

Dr Montserrat Arroyo, WOAH Deputy Director General, International Standards and Science, welcomed members of the Commission, expressing her gratitude for their sustained efforts and contributions over the past 3 years. She highlighted the significance of further elevating the Commission's impact and visibility.

Dr Arroyo updated the Commission on WOAH's standard-setting activities. She noted the harmonisation of processes across the four Commissions that include the new initiative to publish Member comments on draft standards from WOAH's *Manuals* and *Codes*. This initiative reflects WOAH's commitment to transparency and Member engagement. Dr Arroyo also informed the Commission of the schedule for this year's Bureau meetings, involving collaborations between the Aquatic Animal Health Standards and the Biological Standards Commissions, as well as between the Terrestrial Animal Health Standards Commission and Scientific Commission for Animal Diseases, highlighting the organisation's collaborative approach.

Dr Arroyo provided an update on the progress with the Standards Navigation tool, announcing that significant advancements have been made. The tool will be presented to the Assembly during the General Session and is expected to be operational by July 2024.

Turning to WOAH's upcoming events, Dr Arroyo announced that the Commission's pre-General Session webinar is scheduled for Tuesday, 16 April 2024 from 12.00 to 14.00 CET.

Concluding her address, Dr Arroyo expressed appreciation for the Commission's accomplishments during the 3-year term, which included the adoption of 68 chapters, with additional chapters expected to be adopted this year, the implementation of the justification tables for the scores of tests given in Table 1 *Test methods available and their purpose* of the disease-specific chapters, and a strategy to evaluate Reference Centres.

The members of the Commission thanked Dr Arroyo for the excellent support provided by the WOAH Secretariat.

1 ToR: Terms of Reference
2 WHO: World Health Organization

### 1.3. Updates from the WOAH Headquarters

#### 1.3.1. Transparency of the WOAH process for the elaboration of Standards

The Secretariat updated the Commission on progress that had been made to improve the transparency of the WOAH process for the elaboration of Standards, in particular the publication of comments submitted by Members and partners.

The Secretariat informed the Commission that the Director General communicated this initiative to Members in December 2023 and that an SOPs[3] had been developed for the submission of comments during the process for the elaboration of WOAH international standards, as well as a guide on how to submit and present comments, and that these documents have been published on the WOAH website and on the Delegates' website.

The Secretariat reminded the Commission that this is a progressive process that will start in March/April 2024 with the publication on the Delegates' website of comments considered on new and revised standards during February 2024 Commission meetings, at the same time as the publication of the respective February 2024 Commission report. This process takes a step-wise approach and includes an evolution of the Commission reports towards transparency of comments considered and Commission responses, which will result in better documentation and traceability of the WOAH process for the elaboration of Standards.

## 2. Adoption of the agenda

The proposed agenda was presented and adopted. Dr Emmanuel Couacy-Hymann chaired the meeting and the WOAH Secretariat acted as rapporteur. The agenda and the list of participants can be found at Annexes 1 and 2 respectively.

## 3. Collaboration with other Specialist Commissions

### 3.1. Horizontal issues among the Specialist Commissions

#### 3.1.1. Case definitions: tularemia, infection with avian metapneumovirus (turkey rhinotracheitis)

The Biological Standards Commission discussed the case definitions for tularemia and infection with avian metapneumovirus (turkey rhinotracheitis), and gave its recommendations to the Scientific Commission for Animal Diseases (see agenda item 8.3.2. of the report of the meeting of the Scientific Commission for Animal Diseases, 12–16 February 2024).

### 3.2. Scientific Commission for Animal Diseases

Nothing for this meeting.

### 3.3. Terrestrial Animal Health Standards Commission

Matters between the Terrestrial Animal Health Standards Commission and the Biological Standards Commission.

#### 3.3.1. Updates from the September 2023 Code Commission meeting

The Biological Standards Commission was updated by the Secretariat of the Code Commission on the current topics under review by the Code Commission to ensure complementarity and alignment of the two Commission's respective work programmes.

In February 2021, the Code Commission agreed to develop a framework for Terrestrial Code Standards that would serve as a useful guide to ensure standardisation of Terrestrial Code content. Noting the differences in the objectives and structure of the chapters within Volume I and Volume II of the Terrestrial Code, and within the different sections of Volume I, the Commission requested the Secretariat to begin by working on the content of disease-specific chapters, i.e. Volume II.

Since then, Code Commission has worked closely with the Secretariat, in consultation with the Scientific Commission, and based on previous discussions and agreements between the Code Commission, the

3 SOPs: Standard Operating procedure

Scientific Commission and, where relevant with the Biological Standards Commission, to develop a document that provides a detailed description of the structure and content of a disease-specific chapter, including the key references to other parts of the *Terrestrial Code* and other WOAH Standards, and conventions regarding the use of terms, wording and structure.

The Code Commission acknowledged that the framework would be a living document and should be used as the reference for those undertaking work on the development of new or revised chapters. The Commission also agreed that the framework may help Members gain a better understanding of disease-specific chapters in the T*errestrial Code* and could eventually framework be made available to Members at a later stage.

In September 2023, the Code Commission reviewed the document and requested the Secretariat to finalise a first edition for its February 2024 meeting and requested that it be shared at the same time with the Scientific Commission and the Biological Standards Commission. Moreover, the Code Commission requested the Secretariat to use the Framework in upcoming disease-specific chapter revisions and provide feedback.

#### 3.3.2. Biological Standards Commission's recommendations to the Terrestrial Animal Health Standards Commission

See agenda item 5.7. of this report

#### 3.3.3. Update from the Biological Standards Commission on the request from the Code regarding Terrestrial Code Chapter 6.10 Responsible and prudent use of antimicrobial agents in veterinary medicine

See agenda item 5.8. of this report

#### 3.3.4. Question on the chapter on bovine viral diarrhoea

The advice of the Biological Standards Commission was sought regarding the taxonomy of the causative agents of bovine viral diarrhoea. The Biological Standards Commission advised that the taxonomy had been updated and adopted by the International Committee on Taxonomy of Viruses (ICTV). The new nomenclature has been introduced in the *Terrestrial Manual* chapter (see agenda item 5.2) and should be applied to the *Terrestrial Code* chapter:

### 3.4. Aquatic Animal Health Standards Commission

Meeting of the Bureaus of the Commission (see item 3 of the Meeting of the Aquatic Animal Health Standards Commission, 14–21 February 2024.

## 4. Work Programme

The updated work programme was agreed and can be found at Annex 3.

## 5. *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*

For this Agenda Item, the Commission was joined by Dr Steven Edwards, Consultant Editor of the WOAH *Terrestrial Manual*.

### 5.1. Report format and commenting system

In light of the implementation of the new system for submission and publication of Member comments, the Commission reviewed its reporting system. To better report amendments to the *Terrestrial Manual,* the Commission decided to adopt the table format currently used by the Aquatic Animals Commission. Members can more easily see and understand the Commission's decisions in response to comments.

### 5.2. Review of Member comments received on draft chapters and their endorsement for circulation for second-round comment and proposal for adoption in May 2024

The Commission reviewed 15 draft chapters and approved 13 for circulation, some subject to clarification of certain points by the experts, for second-round Member comment before presenting them for adoption by the Assembly in May 2024.

Chapter 1.1.5. 'Quality management in veterinary testing laboratories':

| Section/paragraph | Comment | Decision |
|---|---|---|
| A.2. Standards, guides, and references, paragraph 3 | Move the last sentence to Section A.7.3 *Validation of the test method* | Agree, text fits better in this Section |
| A.3. *Accreditation,* point iii) | Delete the requirement for equipment to be verified and managed in accordance with the relevant maintenance and calibration schedule as not all equipment will need to be verified | Disagree, equipment should be maintained and calibrated following a defined schedule |
| A.6. Quality assurance, quality control and proficiency testing, paragraph 2 | Reinstate the word 'test' in the sentence: 'quality control test-oriented and ensures detection of any problems that arise' | Disagree, the amended sentence is correct: quality control is results-oriented |
| A.7.3.1 Activities that validation might include | Move steps i) and ii) to the end of the list as steps iii) to viii) would be done first as part of a validation process | Agree |

The revised Chapter 1.1.5. 'Quality management in veterinary testing laboratories' is presented as Annex 4 and will be proposed for adoption at the 91st General Session in May 2024.

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

| Section/paragraph | Comment | Decision |
|---|---|---|
| General comment | Include a section for coccidiosis vaccines: the chapter has sections for living viral vaccines, inactivated viral and bacterial vaccines, and living bacterial vaccines, but not a section for live vaccines containing a preparation of sporulated oocysts of a suitable lines of species of coccidial parasites | Agree: this comment will be addressed in the next review cycle (2025/2025) |
| B. Living viral vaccines for administration by injection, or through drinking water, spray, or skin scarification, point 3 | Add the Veterinary Drug Administration of China (People's Rep. of) to the list of acceptable published methods for testing vaccine batches for freedom from extraneous agents | Agree |
| C. Inactivated viral and bacterial vaccines, point 2 | Add 'pre-' before 'inactivated in the sentence: 'If studies on representative extraneous agents are required, then spiking inactivated vaccine with live representative agents and following...' because representative agents should be added to the pre-inactivated vaccine to be inactivated for testing | Disagree: depending on the vaccine it may be safer to work with inactivated vaccine for this test rather than one containing live infectious pathogen |
| G. Protocol examples, Table 1 | Members proposed some minor editorial changes | Agree |
| G.3.2 General testing for exclusion of Mycoplasma sp. | European Medicines Agency's link does not work | Updated the European Medicines Agency's link |

| Section/paragraph | Comment | Decision |
|---|---|---|
| H. Information to be submitted when applying for an import licence, paragraph 1 | Reinstate the requirement that Veterinary Authorities should follow the *Terrestrial Manual* when undertaking risk analysis for biologicals | Agree but clarified that it is the *Terrestrial Code* that should be followed |
| H. Information to be submitted when applying for an import licence, paragraph 2 | Add the Ministry of Agriculture and Rural Affairs of China (People's Rep. of) to the list of examples of a risk-based assessment of veterinary biologicals for import into a country | Agree |

The revised Chapter 1.1.9. 'Tests for sterility and freedom from contamination of biological materials intended for veterinary use' is presented as Annex 5 and will be proposed for adoption at the 91st General Session in May 2024.

Chapter 2.2.4. 'Measurement uncertainty'

| Section/paragraph | Comment | Decision |
|---|---|---|
| *Introduction*, paragraph 2 | For consistency, replace 'cut-off' with 'diagnostic threshold' | Agree |
| A. The necessity of determining MU, paragraph 1 | Replace the term 'confidence interval' with 'reference interval' as it is the correct term used by ISO/IEC Guide 98-3 | Agree and applied this amendment throughout the chapter |
| A. The necessity of determining MU, paragraph 1 | Add a sentence clarifying that alternative methods are available that are less reliant on distributional assumptions, and better handle the presence of outliers | Agree |
| A.2.1 Method of expression of MU | Change the subscript from 'L' to 'W' as 'low' has been changed to 'weak' positive control | Agree and applied this amendment throughout the chapter |
| A.2.1 Method of expression of MU | Define 'X' in the equation and clarify what is meant by transformed result | Agree: added that X represents the set of replicates, and gave examples of a suitably transformed result |
| A.2.3 Calculating uncertainty | | Added a statement on the need to transform not normally distributed data |
| A.2.4 Interpretation of the results | Replace the first sentence with a statement that 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 | Agree, the original interpretation was too precise given the multiple approximations made and the nuances of the interpretation of a reference interval |
| A.3.3 Interpretation of the results | Replace the sentence with a statement that a sample with a Ct between 36 and 37% is within the MU surrounding the threshold value, and thus its diagnostic status is less certain than those of | Partly agree, the original interpretation was too precise given the multiple approximations made and the nuances of the interpretation of a reference interval. However, the threshold is |

| Section/paragraph | Comment | Decision |
|---|---|---|
| | samples with results further from that threshold | 37, the upper limit of the MU is 38 and the lower limit is 36; the values refer to Ct values and thus the percentage sign has been deleted |

The revised Chapter 2.2.4. 'Measurement uncertainty' is presented as Annex 6 and will be proposed for adoption at the 91st General Session in May 2024.

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

| Section/paragraph | Comment | Decision |
|---|---|---|
| Figure 2 | Add 'Infection/disease outcome' and 'Time post-experimental infection' to Column 'Phase of infection data'.<br>Infection/disease outcome is important: while an animal may (or may not) have evidence of infection or clinical signs of disease, the animal may recover. Even if the disease has a high mortality rate, some animals will recover with varying levels of infection or clinical signs, which can create a bias depending on what outcome is being looked for in the diagnostic assay.<br>Time post-experimental infection: this is critical if using reference samples collected from experimental infection models as the analyte will likely change over time. It also allows recreation of samples if the experimental model is repeatable. | Agree |
| F.1 Animals of unknown status – diagnostic specificity and diagnostic sensitivity | Add 'analysis of' between 'Bayesian' and 'latent class models' because latent class is a model and Bayesian is an analysis approach | Agree |

The revised Chapter 2.2.6. 'Selection and use of reference samples and panels' is presented as Annex 7 and will be proposed for adoption at the 91st General Session in May 2024.

Chapter 3.1.5 'Crimean–Congo haemorrhagic fever'

| Section/paragraph | Comment | Decision |
|---|---|---|
| Table 1. Diagnostic test format for Crimean–Congo haemorrhagic fever virus infections in animals, Key | Delete the word 'very' from the key '+ = suitable in very limited circumstances' | Disagree: standard text Table 1 throughout the *Terrestrial Manual* |
| Table 1, real-time RT-PCR method, for the purpose of Individual animal freedom from infection prior to movement | Change the rating from '+++' to '++' due to the transient nature of viremia | Agree. Spengler *et al.* (2016) reviewed research into CCHV and confirms transient viremia |

| Section/paragraph | Comment | Decision |
|---|---|---|
| Table 1, all methods for the purpose of Confirmation of clinical cases in animals | Change the ratings of all the tests in this column to '–' because animals, including ruminants, are typically asymptomatic to infection, although could be transiently viraemic | Disagree: in cases of pyrexia these tests may detect viraemia |
| Table 1, IgM ELISA method, for the purpose of Prevalence of infection – surveillance | Change the rating from '–' to '++' due to the short persistence of IgM antibodies in response to acute infections, but the test has limitations as it may not be detected when IgM wanes | Disagree: the IgM response is weak, and the incidence of a detectable IgM response may be very low in a population given it does not last long. In addition, the IgM ELISA is not designed for use in animals and so has to be adapted prior to use (see Section 2 *Serological tests*) |

The revised Chapter 3.1.5. 'Crimean–Congo haemorrhagic fever' is presented as Annex 8 and will be proposed for adoption at the 91st General Session in May 2024.

Chapter 3.3.6. '*Avian tuberculosis*'

| Section/paragraph | Comment | Decision |
|---|---|---|
| General comments | Change the title of the chapter to avian mycobacteriosis as the disease is nontuberculous | Disagree: the chapter title is based on the pathogenesis of the disease in birds |
| *Summary*, paragraph 3 | Add after 'pet birds owners' 'or caretakers of captive birds' | Agree |
| *Summary*, paragraph 4 | Replace 'gene segments' with 'insertion sequences' as it is more correct, explains the naming convention, and also because some of the insertions are not gene 'segments' – they can contain whole genes, multiple genes, extra repetitive elements, no ORF at all, etc. | Agree |
| *Summary*, paragraph 4 | Include a mention of matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS) as is also a valuable tool. | Agree |
| A. *Introduction*, paragraph 2 | This seems confusing or possibly contains an error. Three species are set out in the initial sentence (*M. avium* subsp. *avium, M. avium* subsp. silvaticum and *M. avium* subsp. paratuberculosis,) and again later three species but not the same three: *M. avium* subsp. avium, *M. avium* subsp. paratuberculosis, and *M. avium* subsp. *Lepraemurium*; and then three sub-species of *M. avium* subspecies avium. In addition, the nomenclature used in the diagnostic section does not seem to include this approach in places, and also refers to additional classifications | Nomenclature of all bacteria is changing very fast. There is a consensus that many of these changes do not affect the treatment of the diseases. New names and classifications take a while to make it into the formal classifications according to the nomenclature standards. In this paragraph, formally approved species are mentioned along with results from recent research. Other sections have the traditional names most clinicians are familiar with and the limitations of typing in resource-limited areas |

| Section/paragraph | Comment | Decision |
|---|---|---|
| | not mentioned here such as serotypes 1,2, and 3 of *M. a. avium* | |
| Table 1. Test methods available for the diagnosis of avian tuberculosis and their purpose | The rating of Ziehl–Neelsen staining for the purpose Confirmation of clinical cases (++) is correct for organ material but not for faecal smears | The text does not refer to faecal smears but only to organs |
| B.1 Identification of the agent | Add a sentence and reference to MALDI-TOF MS as a valuable diagnostic tool | Agree |
| B.1 Identification of the agent | Clarify that though traditionally, M. a. avium is separated from common nonchromogenic slow-growing organisms by their ability to grow at 42°C, the method has limited value, as other species are able to grow at 42°C. | Agree |
| B.1.1 *Culture,* paragraph 1 | Remove commercial names of products | Agree |
| B.1.1 *Culture,* paragraph 4 | Replace the word ‘pet’ with ‘captive’ before ‘birds’ | Agree |
| B.1.2 Nucleic acid recognition methods, paragraph 1 | Correct the presentation of the gene segments by using italics | Agree |
| B.2.1 *Tuberculin test,* paragraph 2 | Add the scientific name ‘(*Phasianus colchicus*)’ after ‘common pheasant’ to avoid confusion between the two different common names for the same species of bird | Agree |
| C.2.2.4, iii) *Safety,* paragraph 1 | The study design in this paragraph is much less specific with regards to number of animals needed, minimum size of the animal and injection volume per animal in contrast to elsewhere in the text | Agee and deleted the last three sentences in the paragraph |
| C.2.2.4, iv) *Batch potency* | For clarity, add ‘shaved (an area large enough’ between ‘flanks’ and ‘to provide space for three-to-four injections on each side).’ | Agree |

The revised Chapter 3.3.6. ‘Avian tuberculosis’ is presented as Annex 9 and will be proposed for adoption at the 91st General Session in May 2024.

Chapter 3.4.1. ‘Bovine anaplasmosis’

| Section/paragraph | Comment | Decision |
|---|---|---|
| General comment | Replace ‘initial bodies’ with ‘inclusion bodies’ throughout the chapter | Agree |
| B.1.1 *Microscopic examination*, paragraphs 1 and 8 | Replace the word ‘parasites’ with ‘bacteria’ | Agree |

| Section/paragraph | Comment | Decision |
|---|---|---|
| Table 1. Test methods available for the diagnosis of bovine anaplasmosis and their purpose | Experts had prepared tables justifying the scores given in Table 1 for the various tests and purposes. Links to these justification tables had been embedded in the Table headings, but they were overlooked by Members during the first round of commenting. | These justification tables have been added as appendices to the chapter and cross referenced in Table 1. |
| Figure 1. Anaplasma marginale *inclusion bodies* | No comment, Commission decision | Request a clearer illustration of inclusion bodies |
| Table 2. Oligonucleotides used in PCR assays to detect A. marginale and A. centrale | Remove the hyphen from the oligonucleotide sequences | Disagree: this is the *Terrestrial Manual* style |
| B.2.2.3 *Data analysis*, last sentence | Replace the word 'reproducibility' with the word 'repeatability' because reproducibility typically refers to inter-laboratory precision. | Agree |

The revised Chapter 3.4.1. 'Bovine anaplasmosis' is presented as Annex 10 and will be proposed for adoption at the 91st General Session in May 2024.

Chapter 3.4.7. 'Bovine viral diarrhoea'

| Section/paragraph | Comment | Decision |
|---|---|---|
| General comment | The taxonomy of the pathogenic agent has been updated. The new taxonomy should be used and applied consistently throughout the chapter: *Pestivirus bovis* (commonly known as BVDV type 1), *Pestivirus tauri* (BVDV type 2), and *Pestivirus brazilense* (BVDV type 3 or Hobi-like pestiviruses) | Agree and implemented this change |
| *Summary*, paragraph 1 | Clarify that bulls may have a prolonged and persistent testicular infection for prolonged periods as the length of the presence of the virus in the testicular tissue could vary significantly from 28 days post-acute infection to 5 years post-infection | Agree |
| *Summary*, paragraph 2 | Add 'or pestivirus A, B, C, D or H', as appropriate, to the pathogenic agents | Disagree, the proposal is not in line with the adopted taxonomy |
| A.1 Impact of the disease, paragraph 2 | Clarify that bulls may have a prolonged and persistent testicular infection and include a reference | Agree |
| Table 1. Test methods available for diagnosis of bovine viral diarrhoea and their purpose | Experts had prepared tables justifying the scores given in Table 1 for the various tests and purposes. Links to these justification tables had been embedded in the Table headings, but they were overlooked by Members during the first round of commenting. | These justification tables have been added as appendices to the chapter and cross referenced in Table 1. |

| Section/paragraph | Comment | Decision |
|---|---|---|
| B.1.1.1 Microplate immunoperoxidase method for mass screening for virus detection in serum samples, Acetone, d) | Add 'antiviral' before 'BVD antibody' to be consistent with the previous method discription | Agree |

The revised Chapter 3.4.7. 'Bovine viral diarrhoea' is presented as Annex 11 and will be proposed for adoption at the 91st General Session in May 2024.

Chapter 3.4.12. 'Lumpy skin disease' (vaccine section only)

| Section/paragraph | Comment | Decision |
|---|---|---|
| A. *Introduction*, paragraph 2 | Replace the subfamily of the pathogenic agent from Chordopoxvirinae to Chordopoxviridae | Disagree, the adopted taxonomy is Chordopoxvirinae |
| B.1.3. Polymerase chain reaction (PCR) | Add two additional real-time PCRs and references | Disagree, only the vaccine section was sent for comment. These proposals can be addressed when the diagnostic test section is updated |

The revised Chapter 3.4.12. 'Lumpy skin disease' (vaccine section only) is presented as Annex 12 and will be proposed for adoption at the 91st General Session in May 2024.

Chapter 3.6.9. 'Equine rhinopneumonitis (infection with Varicellovirus equidalpha1)'

| Section/paragraph | Comment | Decision |
|---|---|---|
| General comment | Update the taxonomy of the pathogenic agent. Clarify the deletion of EHV-4 from the title. | The nomenclature of the virus has changed from equid herpesvirus-1 (EHV-1) to Varicellovirus equidalpha1. The chapter title has been amended and the Code Commission advised of the change. A sentence has been added to stress that the chapter covers EHV-1<br><br>The second part of the title between brackets refers to the title of the corresponding *Terrestrial Code* chapter, as only EHV-1 is listed, that chapter only covers EHV-1 |
| | Replace 'ml' with 'mL' as that is the correct SI symbol | Disagree, both 'mL' and 'ml' are acceptable, the latter is used throughout the *Terrestrial Manual* |
| Summary | Members proposed some minor editorial changes | Agree |
| A. *Introduction,* paragraph 1 | The current taxonomic names of the viruses are: Varicellovirus equidalpha1 and Varicellovirus equidalpha4 | Agree: for the purposes of the chapter, the acronyms EHV-1 and EHV-4 will continue to used |

| Section/paragraph | Comment | Decision |
|---|---|---|
| A. *Introduction,* paragraph 2 | Remove references to EHV-4 throughout the chapter in line with the title | Disagree: it is an important differential, and the relative pathogenic potential of the two viruses is important for diagnosis |
| B. Diagnostic tests, paragraph | A Member proposed some minor editorial changes for clarity | Agree |
| Table 1. Test methods available for the diagnosis of infection with EHV-1 and their purpose | Remove 'equine rhinopneumonitis' from the title of the Table and replace with 'infection with EHV-1' | Agree |
| Table 1 | Experts had prepared tables justifying the scores given in Table 1 for the various tests and purposes. Links to these justification tables had been embedded in the Table headings, but they were overlooked by Members during the first round of commenting | These justification tables have been added as appendices to the chapter and cross referenced in Table 1 |
| Table 1 | Amend the score for the ELISA:<br>from '+' to '++' for Population freedom from infection;<br>from '–' to '++' for Individual animal freedom from infection prior to movement;<br>from '+' to '++' for Confirmation of clinical cases;<br>from '++' to '+++' for Prevalence of infection – surveillance;<br>from '+' to '++' for Immune status in individual animals or populations post-vaccination<br>Amend the score for the CFT:<br>from '+++' to '+' for Confirmation of clinical cases<br>from '+++' to '++' for Immune status in individual animals or populations post-vaccination<br>CFT is more complicated and difficult to maintain then and ELISA, so should not be considered a more suitable test: Hartley *et al.* (2005). Comparison of antibody detection assays for the diagnosis of equine herpesvirus 1 and 4 infections in horses. *Am. J. Vet. Res.*, **66**, 921–928. | Partly agree.<br>The paper by Hartley *et al.* concerns a comparison of antibody detection assays using 33 acute and convalescent serum samples, i.e. it is not a seroprevalence study. The seroprevalence study by El Brini *et al.* (2021) suggests that the ELISA is less sensitive for EHV-1 antibody detection than the VNT.<br>Members are invited to review the explanation for the test scoring in the tables appended to the chapter |
| Table 2, | Delete the first set of primers and probe because of problems with their specificity | Agree |
| B.1.2 Virus detection by polymerase chain reaction, Point of care (POC) molecular tests | Delete this paragraph as it is unusual to refer to methods not fully validated or included in Table 1 | Disagree: the chapter is supposed to be an entry point to the literature, the assays have proven themselves useful, they are only |

| Section/paragraph | Comment | Decision |
|---|---|---|
| | | mentioned briefly. The assays are not included in Table 1 because they are not fully validated |
| B.1.2 Virus detection by polymerase chain reaction, Molecular characterisation | Delete this paragraph: while correct the main point was made in the previous paragraph and the rest is generic. Molecular analysis can be used in every outbreak to support epidemiology | Disagree, it is important to make the point that sequencing cannot reliably predict neuropathogenic strains. The last sentence is provided in the context of the first two sentences, so is appropriate |
| B.2 *Serological tests*, paragraph 1 | Replace 'however' with 'notwithstanding' | Disagree, the term 'notwithstanding' is not common usage and will be confusing to some readers; 'however' is clearer |
| B.2 *Serological tests*, paragraph 4 | A modified live EHV-1 vaccine that lacks the glycoprotein E gene is licensed in Japan, and an ELISA using a synthetic peptide for glycoprotein E as an antigen (Andoh *et al.,* 2013) is used as DIVA[4] for horses vaccinated with this vaccine. Amend the text to take this fact into account | Agree, replaced the last sentence with new text and a reference reflecting the comment |
| C.2.1.3 Validation as a vaccine strain | Include a quantitative measure on the upper limit of VNT titre (serological status) of horses used to confirm immunogenicity of Master Seed Virus for vaccines. The rationale is that it will be very hard if not impossible to find immunologically naïve horses for this test | Agree and included a reference |
| C.2.3.4 *Duration of immunity*, paragraph 2 | Acknowledging that EHV-1 and EHV-4 are cross reactive, but it is confusing to imply that there are two agents being discussed here called EHV-1 and EHV1/4. It would be clearer to either say 'EHV-1 or EHV-4' or to remove the mention of EHV-4 altogether | Agree to remove mention of EHV-4 here |

The revised Chapter 3.6.9. 'Equine rhinopneumonitis (infection with Varicellovirus equidalpha1)' is presented as Annex 13 and will be proposed for adoption at the 91st General Session in May 2024.

Chapter 3.8.1. 'Border disease'

| Section/paragraph | Comment | Decision |
|---|---|---|
| *Summary*, paragraph 3 | Add mention of pestivirus A, B, C, D or H, as appropriate, to the pathogenic agents | Disagree, the proposal is not in line with the adopted taxonomy |
| A. *Introduction*, paragraph 1 | Update the information on the genotypes and add more details and a reference | Agree, the detail is necessary as BDV requires differential diagnosis from CSFV |

4 DIVA: differentiate infected from vaccinated animals

| Section/paragraph | Comment | Decision |
|---|---|---|
| B.2.1.1 *Test procedure*, iii) | It is not clear why the acceptance limits were changed. They should be consistent with the BVD chapter re currently | Agree, the original range of 30–300 $TCID_{50}$ is reinstated: acceptable ranges are either calculated by the Reed and Muench or Spearman and Kärber methods |
| C.1.1 Characteristics of a target product profile, paragraph 1 | Replace ‘afford’ with ‘provide’ and ‘fetal infection’ with ‘fetal protection’ | Agree |

The revised Chapter 3.8.1. ‘Border disease’ is presented as Annex 14 and will be proposed for adoption at the 91st General Session in May 2024.

Chapter 3.8.12. ‘Sheep pox and goat pox’:

| Section/paragraph | Comment | Decision |
|---|---|---|
| A. *Introduction*, paragraph 1 | Replace ‘fully susceptible’ with ‘naïve’ | Agree |
| A. *Introduction*, paragraph 7 | Add a sentence that there is no evidence of persistently infected animals, and more details on characteristics of the virus, i.e. resistance to physical and chemical actions | Agree |
| B.1.1 Specimen collection and submission, paragraphs 1 and 3 | Delete antigen detection for consistency with Table 1 | Agree |
| B.1.1 Specimen collection and submission, paragraph 1 | Add a sentence stating that nasal and buccal swabs can also be collected because the virus will be present in nasal and saliva discharges | Agree |
| B.1.1 Specimen collection and submission, paragraph 2 | Delete the statement that tissues in formalin have no special transportation requirements as it is vague and misleading. Sample submission should be described in the introductory chapter | Agree |
| B.1.2 Virus isolation | Replace ‘antigen detection’ with ‘genome detection’ | Agree |
| B.1.4 Histopathology | Delete ‘and mounting of the formalin-fixed biopsy material’ from the second sentence: the sequence is incorrect, incomplete, and unnecessary as this is a routine procedure and not specific to sheep pox. The previous sentence is sufficient | Agree |
| B.1.6 Nucleic acid recognition methods, paragraph 1 | Add blood and semen a sample types | Agree and added a sentence to clarify that nucleic acid extraction and PCR amplification methods must be validated for the sample matrix being tested |
| B.1.6.2 *Real-time PCR methods*, paragraph 1 | Add a reference to the list of pan-capripox virus real-time PCR assays: | Agree |

| Section/paragraph | Comment | Decision |
|---|---|---|
| | the test is used by the EURL for capripox viruses and several other national reference laboratories from Europe, and all the validation information on this test can be found in the publication | |
| B.1.6.2 *Real-time PCR methods*, paragraph 2 | Clarify that the method is for the detection of genomic DNA | Agree |
| B.1.6.2 *Real-time PCR methods*, DNA extraction from blood and tissue | Clarify that commercially available kits are for the extraction not isolation of DNA, and that the manufacturer's instructions should be followed | Agree |
| B.1.6.2 *Real-time PCR methods*, Real-time PCR, iii) and iv) | Clarify that any commercial real-time PCR kit of choice can be used | Disagree, does not add any value to the test description |
| B.1.6.3 Isothermal genome amplification | Clarify that LAMP assays were reported to differentiate GTPV from SPPV | Agree |
| B.2 Serological tests | Add a sentence that blood for antibody detection should be collected in tubes without anticoagulant | Disagree: self-evident |
| B.2 Serological tests | Add text that detectable levels of antibodies develop 1 week after the animal shows clinical signs. The highest antibody levels are detected within 1–2 months after infection is detected | Agree |
| C.1.1 Rationale and intended use of the product | Add information on live attenuated vaccines | Disagree, only the diagnostic tests section was sent for comment. This proposal can be addressed when the vaccine section is updated |

The revised Chapter 3.8.12. 'Sheep pox and goat pox' is presented as Annex 15 and will be proposed for adoption at the 91st General Session in May 2024.

Chapter 3.9.1. 'African swine fever (infection with African swine fever virus)' (vaccine section only)

A large number of comments were received on the newly proposed vaccine section. Given that live modified vaccines are in use in some Members, the Commission believes it is important to have a minimum standard in the WOAH *Terrestrial Manual*, with the commitment to review it regularly as scientific evidence becomes available.

| Section/paragraph | Comment | Decision |
|---|---|---|
| General comment | Some Members have reservations about including vaccine standards in the *Terrestrial Manual* because of safety issues in the field | The Commission is aware of these issues, and of the fact that vaccines are currently authorised by some national regulatory authorities and in use in the field. The proposed Section was drafted by experts in liaison with vaccine manufacturers and veterinary medicine regulatory experts. It addresses these issues as best it can based on current scientific evidence. The Commission has a |

| Section/paragraph | Comment | Decision |
|---|---|---|
| | | strong position that it is better to offer to national authorities and vaccine manufacturers science-based recommendations on ASF vaccines in the chapter rather than none at all |
| Summary | Some Members had provided comments on the *Summary* | Only the vaccine section was sent for comment. These proposals can be addressed when the diagnostic test section is updated |
| A. Introduction | Some Members had provided comments on unmodified text in the *Introduction* | Only the vaccine section was sent for comment. These proposals can be addressed when the diagnostic test section is updated |
| A. *Introduction,* paragraph 9 | Amend the modified text to include information on mutants and recombinants that have emerged with potentially increasing prevalence, along with two references | Agree, text added |
| A. *Introduction,* paragraph 9 | Add a sentence stating that it is not always necessary to follow the principles given in the Chapter 1.1.8 *Principles of veterinary vaccine production* when there are scientifically justifiable reasons for using alternative approaches | Disagree, chapter 1.1.8 is an adopted Standard and not an example |
| A. *Introduction,* paragraph 9 | Add a paragraph stating that it is crucial to confirm the absence of circulating strains of other ASFV genotypes before the use of the vaccine due to the characteristics of ASFV, where frequent recombination occurs between different strains. And add a statement that it is essential to establish a robust vigilance monitoring system to rapidly detect and notify unexpected events resulting from such recombinations | Agree that it is important to confirm what genotypes of ASFV are circulating in a population prior to vaccination: added a sentence to C.1. Background, paragraph 16. Also agree it is important to have a robust monitoring system. Text amended accordingly |
| A. *Introduction,* paragraph 9 | Reword the sentence on the validation of modified live vaccines (MLV) to remove non-transmissibility as according to the minimum standard some virus vaccine transmission might be allowed | Agree |
| A. *Introduction,* paragraph 10 | Include safety and efficacy in different age-groups of pigs, including breeding boars and pregnant sows | The Commission's position is that such tests are preferred but not required in the minimum standard. At present, no vaccine is used in pregnant sows |
| A. *Introduction,* paragraph 10 | Include duration of immunity and onset of immunity in the minimum standard | Agree, a statement has been added that onset and duration of immunity are also required to meet minimum standards |

| Section/paragraph | Comment | Decision |
|---|---|---|
| C.1. *Background*, paragraph 1 | Add a sentence on the prevalence of other genotypes and recombinants | Agree and amended the proposed text |
| C.1. *Background*, paragraph 3 | Clarify that the appropriate biosecurity level should be based on the virulence and characteristics of the virus | Disagree, the concept is covered by the word 'appropriate' |
| C.1. *Background*, Safe | Add reference to the definition of fever | Agree |
| C.1. *Background*, Safe and Efficacious | Minimum requirements for MLV should include safety for pregnant sows, pigs at different growth stages (suckling piglets, nursery pigs, fattening pigs), breeding boars, and cross-protection against other strains currently circulating in the field. | The Commission reiterated that its position is that such tests are preferred but not required in the minimum standard. The test would be needed if the MLV is to be licensed for those subpopulations |
| C.1. *Background*, Efficacious | Add 'ASF-induced' before 'mortality' | Disagree, the meaning is implicit |
| C.1. *Background*, Quality – potent | Replace 'potent' with 'stability' | Agree |
| C.1. *Background*, Quality – Identity | Replace 'identity' with 'vaccine matching | Agree |
| C.1 *Background*, paragraph 9 | Include a statement that more research is needed to determine whether these genotype 2-specific MLVs can effectively protect against newly circulating variants of genotype II and recombinant strains | Agree |
| C.1 *Background*, paragraph 10 | Include the target species of the vaccines that have been authorised | Agree |
| C.1 *Background*, paragraph 11, fifth point | Add a reference to a new vaccine candidate strain that has been demonstrated to provide strong suppression of viremia, etc., thus is expected to have higher safety compared with the virus in previous studies | Agree |
| C.1 *Background*, paragraph 11 and all points | There is a possibility that an animal that inadvertently received two different vaccine strains (with different single gene deletions) could potentially regenerate a fully virulent ASFV by recombination. Consider requiring that all MLV ASFV vaccines have at least one attenuating deletion in common so that it is not possible for this to occur. Also consider combining these viruses: all were designed by homologous recombination and are deletion mutants with a different number of genes deleted | This is a possibility but the risk of reversion by recombination through co-infection with a vaccine and wild-type strain is significantly higher. Requiring vaccines to have a single gene in common would be technically challenging considering that biological basis for attenuation remains poorly understood |
| C.1 *Background*, paragraph 13 | The text makes it sound like the next generation vaccines will be MLVs; it is | Disagree, the text existing is clear |

| Section/paragraph | Comment | Decision |
|---|---|---|
| | likely other technology will be better. This wording will create confusion through the vaccine section | |
| C.1 *Background*, paragraph 13 | Add a statement that there is no inactivated vaccine with any level of protection that could be acceptable | Disagree, the existing text is clear |
| C.1 *Background*, paragraph 15 | Delete MLV as this should apply to new vaccine technology developed | Agree |
| C.1 *Background*, paragraph 15 | Add a description emphasising the importance of pharmacovigilance for ASF vaccine | Agree |
| C.1 *Background*, paragraph 16 | Clarify the definition of ‘exceptional circumstances’ | Agree |
| C.2.1.2 Quality criteria (sterility, purity, freedom from extraneous agents) paragraph 1 | Delete the last sentence as it is not in the appropriate place. The safety requirements are explained elsewhere. | Agree |
| C.2.1.2 Quality criteria (sterility, purity, freedom from extraneous agents) paragraph 3 | Include a more detailed explanation of the reason why genetic stability to at least MSV+10 should be demonstrated when MSV+8 is the maximum passage for use in final product manufacturing | Agree, clarified that if final product yields are low, demonstration of stability is required for the maximum passage for use in the final product manufacturing as defined by the producer |
| C.2.2.4 Final product batch tests, ii) identity | Clarify the goal of the parenthetical (e.g. specific differential real-time PCR) | Agree, added that the detection methods should also differentiate the vaccine virus from the parent strain of the virus as a potential contaminant |
| C.2.2.4 *Final product batch tests,* vi) Residual humidity/residual moisture | Delete mention of the route of administration as the test will be required for any lyophilised or freeze-dried vaccine regardless of the route of administration | Agree |
| C.2.3.1 Manufacturing process | Amend the sentence as it is not necessary to provide information on consecutive vaccine batches and obtaining information from three or more batches is preferable | Agree, the text is deleted |
| C.2.3.2 Safety requirements | This Section is very detailed and prescriptive. Can it be shortened or added to another Annex? Suggest focus is on the principles rather than the exact nature on how to conduct the experiment | Disagree, general principles are given in chapter 1.1.8, the details given here are specific to ASF |
| C.2.3.2 Safety requirements | Additional demonstration of MLV safety in breeding age gilts and pregnant sows is preferred but not required as a minimum standard | Agree as before. Current vaccines are not licensed in breeding animals. Demonstration of safety in breeding age gilts and pregnant sows should be required if the vaccine is intended to be used in those subpopulations. This |

| Section/paragraph | Comment | Decision |
|---|---|---|
| | | standard will be regularly reviewed as more data become available on the use of these vaccines |
| C.2.3.2 *Safety requirements,* i) Safety in young animals | Unless the most sensitive category for safety testing is considered to be pigs of 6–10 weeks of age, a more flexible wording would be preferable | Agree, amended to a minimum of 4 weeks and not older than 10 weeks in accordance with current evidence |
| C.2.3.2 *Safety requirements,* i) Safety in young animals, paragraph 5 | The monitoring period proposed is far longer than that proposed in the VICH GL44 for target animal safety for veterinary live and inactivated vaccines. When injection site adverse reactions are present at the end of the 14 days observation, the observation period should be extended until clinically acceptable resolution of the lesion has occurred or, if appropriate, until the animal is euthanised, and histopathological examination is performed | Disagree, the period given here is to cover the chronic clinical signs that can appear many weeks after vaccination. This is not related to injection site adverse reactions |
| C.2.3.2 *Safety requirements,* i) Safety in young animals, paragraph 5 | To detect any potential virus shedding, add checks for vaccine virus in oral, nasal and faecal secretions, every 7 days for at least 60 days and checks for vaccine virus in tissues at 28 days, | Disagree because there may be limited shedding associated with the use of MLV vaccines. Introducing a requirement for no shedding will preclude the use of vaccine. Despite being considered safe, a minimum level of horizontal transmission may be expected for MLV |
| C.2.3.2 *Safety requirements,* i) Safety in young animals, paragraph 7, first point | Clarify that no piglet shows notable signs of disease: current wording could be interpreted as if vaccinated piglets showing notable signs of disease but not reaching the pre-determined humane endpoint would comply with the test | Agree |
| C.2.3.2 *Safety requirements,* i) Safety in young animals, paragraph 7, second point | Clarify what is meant by ‘average’ body temperature increase | Agree, the point has been rewritten |
| C.2.3.2 *Safety requirements,* i) Safety test in pregnant sows and test for transplacental transmission, paragraph 1 | As MLV itself can infect sows through horizontal transmission and can vertically transmit to fetal pigs, delete the current text and replace it with the requirement to test sows and the farrowings for virus shedding | Disagree: as above, the current text is correct; additional testing should only be required if the manufacturer recommends the use of the vaccine in breeding-age sows and pregnant sows |
| C.2.3.2 *Safety requirements,* ii) Safety test in pregnant sows and test for transplacental transmission, paragraph 1 | Correct the first sentence as there have been experimental studies looking at the transmission of a genotype II ASFV from pregnant sows to the fetuses | Agree |
| C.2.3.2 *Safety requirements,* iii) Horizontal transmission, paragraph 1 | Amend ‘no fewer than 12 healthy piglets’ to ‘healthy piglets in sufficient number to confirm the presence or | Disagree, this text is consistent with CSF chapter and it is applicable to ASF |

| Section/paragraph | Comment | Decision |
|---|---|---|
| | absence of horizontal transmission between vaccinated animals and naïve animals' as there is no scientific basis for using 12 piglets. | |
| C.2.3.2 *Safety requirements,* iii) Horizontal transmission, paragraph 1 | Consider not to co-mingle directly. If oral vaccines are considered, environmental contamination with vaccine virus could lead to 'vaccination' of naïve contacts | This point is valid and will be put forward for consideration when oral vaccines are ready for testing |
| C.2.3.2 *Safety requirements,* iii) Horizontal transmission, paragraph 4 | No comment, arose from discussion with experts | Clarified what is meant by body temperature increase here and throughout the chapter, where appropriate |
| C.2.3.2 *Safety requirements,* iii) Horizontal transmission, paragraph 5 | Delete the requirement to determine infectious virus titres by quantitative virus isolation | Disagree: virus genome persists for a much longer time than infectious virus so use of PCR alone can give misleading results. It is important to measure infectious virus (PCR may be used to identify samples that may potentially have infectious virus). PCR-positives should also be tested by qualitative/quantitative virus isolation |
| C.2.3.2 *Safety requirements,* iii) Horizontal transmission, paragraph 8, third point | Clarify the vaccine acceptance criteria | Agree and clarified the points: the Commission acknowledges that, according to current evidence, a minimum horizontal transmission could be expected for MLV yet the vaccine could be considered safe |
| C.2.3.2 *Safety requirements,* iv) Post-vaccination kinetics of viral replication (MLV blood and tissue dissemination) study, paragraph 8 | Extend the days on which to euthanise piglets and determine virus titres to include Days 1, 3, and 5 | Partly agree: will include day 5. Day 3 possible with highly virulent virus but if attenuated could be later; days 1 and 3 likely to be negative |
| C.2.3.2 *Safety requirements,* v) Reversion to virulence, First passage (p1), paragraph 1 | Clarify the observation parameters | Agree, amended to be consistent with the agreed standard text here and throughout the chapter, where appropriate |
| C.2.3.2 *Safety requirements,* v) Reversion to virulence, First passage (p1), paragraph 3 | Extend the days on which to euthanise piglets and determine virus titres to include Days 1, 3, and 5 | As before, partly agree: will include day 5. Day 3 possible with highly virulent virus but if attenuated could be later; days 1 and 3 likely to be negative |
| C.2.3.2 *Safety requirements,* v) Reversion to virulence, second passage (p2), paragraph 2 | For consistency, change 'intramuscular administration' to 'intended route' | Agree |
| C.2.3.2 *Safety requirements,* v) Reversion to virulence, Fifth passage (p5), paragraph 3, second point | Clarify what is meant by the term 'minimal chronic' clinical signs | Agree, amended the text to refer to mild clinical signs |

| Section/paragraph | Comment | Decision |
|---|---|---|
| C.2.3.2 *Safety requirements,* v) Reversion to virulence, Fifth passage (p5), paragraph 4 | Delete paragraph, the requirements may not be feasible for countries where the disease is not endemic | Disagree, misunderstanding of the text. Field testing is very important and can be done in an endemic country. It need not necessarily be done in every country that wishes to use the vaccine as long as the relevant data is made available from the countries where the field testing has been carried out |
| C.2.3.3 *Efficacy requirements*, i) Protective dose, paragraph 3 | Propose to replace the description, which is not in line with the VICH guidelines, with a more feasible description. There is no specification in the VICH guidelines regarding the test setting for protective dose such as number of pigs kept, age, consistency of origin, composition | Disagree: VICH does not provide a specific protocol for determining the minimal protective dose. However, the text is in line with general guidelines for safety testing as described in VICH GL 44 and other prescriptive documents. Protective dose is one of the defining characteristics of vaccines and will be required by most if not all Regulating Authorities |
| C.2.3.3 *Efficacy requirements*, i) Protective dose, paragraph 5 | Amend the text so that the animal challenge tests are conducted using all the circulating strains present in the field | Disagree, not suitable for a minimal standard |
| C.2.3.3 *Efficacy requirements*, i) Protective dose, paragraph 5 | Delete 'or non-HAD viruses' $HAD_{50}$ or $TCID_{50}$ are indistinguishable | Disagree, maintain the original text |
| C.2.3.3 *Efficacy requirements*, i) Protective dose, paragraph 6 | Add oral, nasal and anal to the samples to be collected from vaccinated challenged piglets and test every 7 days for 60 days | Partly agree: added the samples but limited the observation period to at least 45 days and preferably 60 days post-challenge |
| C.2.3.3 *Efficacy requirements*, i) Protective dose, paragraph 8 | Add histopathology after gross pathology | Agree |
| C.2.3.3 *Efficacy requirements*, i) Protective dose, paragraph 9 | 100% mortality and morbidity may not be possible depending on the strain used for the experimental infection, i.e. not all strains will cause 100% mortality in control pigs. It is better to design the study to have some flexibility so: a) the experimental infection is repeatable and b) an appropriate number of animals are used to ensure statistically relevant findings are made regarding if the vaccine is providing protection | Agree, amended the text |
| C.2.3.3 *Efficacy requirements*, i) Protective dose, paragraph 10, second point | Clarify what is meant by average body temperature increase | Agree and amended here and throughout the chapter, where appropriate |
| C.2.3.3 *Efficacy requirements*, ii) Assessment for horizontal transmission (challenge virus shed and spread study), paragraph 10 | Add days 7 and 14 to the days on which blood samples will be collected from naïve contact pigs and extend the observation period to 2 months due to | Partly agree, antibodies may not be present in the contact pigs on days 7 and14, post-contact, so better that blood samples are tested for antibodies on day 21 and 28 and at the end of the test |

| Section/paragraph | Comment | Decision |
|---|---|---|
| | the likely low dose of infection in the naïve contact pigs | period. Agreed to extend the observation period to at least 60 days, preferably 2 months |
| C.2.3.3 *Efficacy requirements*, ii) Assessment for horizontal transmission (challenge virus shed and spread study), paragraph 12 | Add the requirement to make histopathological sections and check the cytopathic situation, and to undertake immunohistochemistry tests of ASFV to further trace its distribution in the organs | Disagree, the object is not to characterise the virus, PCR testing of the tissues is sufficient |
| C.2.3.4 Duration of immunity | Add a section on vaccine recombination: the MLV may have genetic recombination with the circulating strains in the field and other vaccine strains. It is thus recommended to carry out vaccine recombination experiments to evaluate the risk of vaccine recombination | Partly agree as it is documented that recombination can occur but, due to the difficulty in carrying out these types of 'recombination' studies in the laboratory, this should not be a minimum standard, but could be a recommendation. Added text to C. Background, paragraph 16 |

The newly drafted section of Chapter 3.9.1. 'African swine fever' (infection with African swine fever virus) (vaccine section only) is presented as Annex 16 and will be proposed for adoption at the 91st General Session in May 2024.

| | Appendix | Chapter | |
|---|---|---|---|
| 1. | 4 | 1.1.5. | Quality management in veterinary testing laboratories |
| 2. | 5 | 1.1.9. | Tests for sterility and freedom from contamination of biological materials intended for veterinary use |
| 3. | 6 | 2.2.4. | Measurement uncertainty |
| 4. | 7 | 2.2.6. | Selection and use of reference samples and panels |
| 5. | 8 | 3.1.5. | Crimean–Congo haemorrhagic fever |
| 6. | 9 | 3.3.6. | Avian tuberculosis |
| 7. | 10 | 3.4.1. | Bovine anaplasmosis |
| 8. | 11 | 3.4.7. | Bovine viral diarrhoea |
| 9. | 12 | 3.4.12. | Lumpy skin disease (vaccine section only) |
| 10. | 13 | 3.6.9. | Equine rhinopneumonitis (infection with Varicellovirus equidalpha1) |
| 11. | 14 | 3.8.1. | Border disease |
| 12. | 15 | 3.8.12. | Sheep pox and goat pox |
| 13. | 16 | 3.9.1. | African swine fever (vaccine section only) |

### 5.3. Fast-track revision of the chapter on avian influenza: follow-up from the Animal Health Forum and the adopted Resolution on avian influenza

At the meeting in September 2023, the Commission agreed on the need for a fast-track review of the *Terrestrial Manual* chapter on avian influenza to ensure the information is up to date with the latest science and fit for purpose. To this end, the WOAH Reference Laboratories were asked to update the chapter to include important amendments as needed on an immediate basis. The objective was to submit the chapter for one review round with the February 2024 report and propose it for adoption in May 2024.

The Commission noted that the update submitted by the Reference Laboratories had been extensively amended. The idea of the fast-track review was to include minimal essential revisions, such as primer and probe sequences, that are urgently required to keep the chapter valid in the current context of avian influenza situation and that could be proposed for adoption after just one commenting round. The Commission decided that for a chapter that had been substantially revised, more than one commenting round was required before submission to the Assembly and so

agreed to put the update in the 2024/2025 review cycle where it would follow the normal review procedure (two rounds of commenting in October and March before being proposed for adoption in May 2025). The Reference Laboratories would have the opportunity to further amend it before re-submission to WOAH in July 2024.

In the meantime, the Commission was made aware of a booklet entitled 'Protocols and guidelines for ASF', which had been developed by the ASF network and would be made available on the WOAH website in the near future. The Commission agreed that it is an excellent method of publishing essential updates to protocols, including primer and probe sequences, rapidly with widespread dissemination of vital information. The OFFLU network would be asked if they could develop a similar publication for avian influenza.

### 5.4. Update on Chapter 2.3.1 The application of biotechnology to the development of vaccines for veterinary use

The Commission identified a Collaborating Centre and expert that could assist with the revision of this chapter. The Commission would like to include future research approaches in vaccine development while retaining information on classical vaccine developments. Their proposal is that the chapter should focus on vaccines against the WOAH listed diseases including:

1. Classical vaccines

2. New generation vaccines

3. Future research approaches in vaccine development.

### 5.5. Update on draft chapter on diagnostic validation of point-of-care tests for WOAH-listed viral diseases using field samples

Since the last meeting, the ASF, rabies and peste des petits ruminants (PPR) Reference Laboratory networks have been asked to comment on the draft of a new chapter on the diagnostic validation of point-of-care tests (POCTs) for WOAH-listed viral diseases using field samples. The networks agree with the principle of publishing information on validation of POCTs, whether as a stand-alone chapter or part of Chapter 1.1.6 or disease-specific chapters, but felt the text needed further development to improve its practicality and applicability. The comments would be submitted to the Collaborating Centre expert who had drafted the text to decide on the best way forward.

### 5.6. Progress on development of a validation report form for tests recommended in the *Terrestrial Manual*

The validation report template has now been finalised and is available on the Commission's web page for contributors to the *Terrestrial Manual* to provide data regarding the tests they recommend.

### 5.7. Application of the criteria for keeping chapters in the *Terrestrial Manual* on non-listed diseases

There are currently 26 chapters in the *Terrestrial Manual* for non-listed diseases. Some of these chapters are for delisted diseases that no longer fulfil the listing criteria (e.g. leptospirosis), and others are for diseases, often zoonoses, that were never listed but for which it was deemed important to provide Members with diagnostic information (e.g. toxoplasmosis) The Commission is aware that maintaining these chapters may not be the best use of resources, and noted that for some there are no designated Reference Laboratories, which poses problems for maintaining the chapters up to date. The Commission agreed the following evidence-based criteria be applied when deciding to maintain a chapter in the *Terrestrial Manual* on non-listed diseases:

1. An important differential diagnosis for a listed disease
2. A Reference Laboratory for the disease exists and is able to provide scientific support
3. A *Terrestrial Code* chapter exists

The Commission applied these criteria to the current 26 non-listed disease chapters in the *Terrestrial Manual.*

The following chapters were retained:

| 1. | Leptospirosis | 2. | Hendra virus disease |
|---|---|---|---|
| 3. | Vesicular stomatitis | 4. | Marek's disease |
| 5. | Border disease | 5. | Melioidosis |
| 7. | Influenza A viruses of swine | 8. | Swine vesicular disease |
| 9. | Verocytotoxigenic *Escherichia coli* | 10. | Bunyaviral diseases of animals (excluding Rift Valley fever and Crimean-Congo haemorrhagic fever) |
| 11. | Zoonoses transmissible from non-human primates | | |

The following chapters will be removed from the next edition after the General Session in May 2024. These chapters will still be available from the BSC secretariat (BSC.Secretariat@woah.org) upon request:

| 1. | Nosemosis of honey bees | 2. | Avian tuberculosis* |
|---|---|---|---|
| 3. | Duck virus enteritis | 4. | Fowl cholera |
| 5. | Fowl pox | 6. | Malignant catarrhal fever |
| 7. | Epizootic lymphangitis | 8. | Ovine pulmonary adenocarcinoma (adenomatosis) |
| 9. | Atrophic rhinitis of swine | 10. | Teschovirus encephalomyelitis |
| 11. | Cryptosporidiosis | 12. | Infection with *Campylobacter jejuni* and *C. coli* |
| 13. | *Listeria monocytogenes* | 14. | Mange |
| 15. | Toxoplasmosis | | |

*once the chapter is adopted in May 2024, information on avian tuberculin will be moved to the mammalian tuberculosis chapter and this chapter will be removed

The Commission also agreed that once these chapters have been removed from the *Terrestrial Manual* in May, it would no longer accept applications for Reference Laboratories for non-listed diseases.

### 5.8. Review of advice submitted by experts of seven *Terrestrial Manual* chapters updated and circulated in October 2023 on whether the update had an impact on the corresponding chapter in the *Terrestrial Code*

At the September 2022 meeting of the Bureaus of the Code and Biological Standards Commissions, it was agreed that the experts who reviewed a *Terrestrial Manual* chapter be requested to advise the Biological Standards Commission as to whether the proposed revision could have an impact on the corresponding *Terrestrial Code* chapter. Six *Terrestrial Manual* chapters in the current review cycle were identified as having a potential impact on the *Terrestrial Code.* The Biological Standards Commissions reviewed the advice received from experts who had undertaken the updates and agreed to submit the following recommendations to the Code Commission:

| Code chapter | Recommendations from Biological Standards Commission to the Code Commission |
|---|---|
| Chapter 11.1. Bovine anaplasmosis | The Commission agrees that Article 11.1.2 of the *Terrestrial Code* chapter could be updated to take account of improved diagnostic test methods and knowledge of the effective treatment methods |
| Chapter 11.X. Bovine viral diarrhoea | The Commission agrees that the taxonomy of the agent in the *Terrestrial Code* should be updated to align with the *Terrestrial Manual* |
| Chapter 11.9. Lumpy skin disease | The Commission agrees the *Terrestrial Manual* update has no impact on the *Terrestrial Code* chapter |

| Code chapter | Recommendations from Biological Standards Commission to the Code Commission |
|---|---|
| Chapter 12.8. Equine rhinopneumonitis | The Commission agrees that the taxonomy of the agent in the *Terrestrial Code* should be updated to align with the *Terrestrial Manual.* It would also be useful to add a case definition to the *Terrestrial Code* |
| Chapter 14.9. Sheep pox and goat pox | The Commission agrees the *Terrestrial Manual* update has no impact on the *Terrestrial Code* chapter |
| Chapter 15.1. Infection with African swine fever virus | The Commission agrees that the *Terrestrial Code* chapter should be updated due to the inclusion of vaccination in the *Terrestrial Manual* |

### 5.9. Update on the request from the Code Commission regarding Chapter 2.1.1 Laboratory methodologies for bacterial antimicrobial susceptibility testing

The Commission was updated on progress since the September 2023 meeting on the request from the Code Commission to review *Terrestrial Manual* Chapter 2.1.1. *Laboratory methodologies for bacterial antimicrobial susceptibility testing* to determine if the chapter provides sufficient and up-to-date information on the establishment of clinical breakpoints or whether it needs to be revised.

To address this request, the Biological Standards Commission consulted the WOAH Working Group on Antimicrobial Resistance (AMR). The Group advised that the expertise on current and upcoming laboratory methodologies for AMR, including the establishment of clinical breakpoints, sits with WOAH's Collaborating Centres. In October 2023, three relevant WOAH Collaborating Centres were asked to review the current chapter and submit a detailed outline of what needs to be done to update it and address the Member concerns.

The Commission reviewed the 'map' submitted by the Centres and agreed the planned outline for the chapter's revision. The Centres would be asked to implement their plan and submit the updated chapter for review at the September 2024 meeting. The aim is to propose the chapter for adoption in May 2025.

### 5.10. Request to reconsider inclusion of foot and mouth disease virus-like particles in the WOAH *Terrestrial Manual*

A group of researchers that had developed a novel foot and mouth disease (FMD) vaccine based on recombinantly expressed virus-like particles (VLP) requested that the Commission reconsider its decision not to include VLP vaccines in the *Terrestrial Manual* until they have received market authorisation (cf: Report of the Meeting of the Biological Standards Commission/September 2022). The Commission again consulted the WOAH Reference Laboratories for FMD, and again concluded that it is too early for such an addition to the *Terrestrial Manual*, which does not include vaccines not yet in use. Given the significance of such vaccines, including their impact on the *Terrestrial Code*, the Commission would welcome reports from the developers on progress with the registration process, and any peer-reviewed information on their use. Once the vaccines are available and in use, the Commission could implement the fast-track procedure to include them in the *Terrestrial Manual* should the Reference Laboratory network of experts deem it appropriate.

### 5.11. Follow-up from the General Session: proposal to include a vaccine in the chapter on American foulbrood

At the General Session, a Member had informed the Assembly that a new vaccine for *Paenibacillus larvae* had been authorised for use in the country and requested that the vaccine be included in Chapter 3.2.2. '*American foulbrood of honey bees* (*infection of honey bees with* Paenibacillus larvae)'. The WOAH Reference Laboratories advised the Commission that the vaccine remains under study and thus, does not yet have the necessary scientific support to warrant a recommendation for its inclusion in the *Terrestrial Manual*. The Commission will request the experts to monitor the field trials and inform the Commission if the vaccine can be included in the future.

### 5.12. Terrestrial *Manual* status: update on chapters selected for the 2024/2025 review cycle

The Commission encouraged those Reference Laboratories with outstanding chapters to deliver by the deadline. The following chapters have been identified for update in 2024/2025 (year last adopted in brackets after the title).

1.1.2. Collection, submission and storage of diagnostic specimens (2013)

1.1.3. Transport of biological materials (2018)

1.1.4. Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities (2015)

1.1.7. Standards for high throughput sequencing, bioinformatics and computational genomics (2016)

2.1.3. Managing biorisk: examples of aligning risk management strategies with assessed biorisks (2014)

2.1.1. Laboratory methodologies for bacterial antimicrobial susceptibility testing (2019)

2.2.1 Development and optimisation of antibody detection assays (2014)

2.2.2 Development and optimisation of antigen detection assays (2014)

2.2.3 Development and optimisation of nucleic acid detection assays (2014)

2.2.5 Statistical approaches to validation (2014)

2.2.7 Principles and methods for the validation of diagnostic tests for infectious diseases applicable to wildlife (2014)

2.2.8. Comparability of assays after minor changes in a validated test method (2016)

2.3.2. The role of official bodies in the international regulation of veterinary biologicals (2018)

2.3.3. Minimum requirements for the organisation and management of a vaccine manufacturing facility (2016)

2.3.5. Minimum requirements for aseptic production in vaccine manufacture (2016)

3.1.2. Aujeszky's disease (infection with Aujeszky's disease virus) (2018)

3.1.8. Foot and mouth disease (infection with foot and mouth disease virus) (2021)

3.1.9. Heartwater (2018)

3.1.14. New World screwworm (*Cochliomyia hominivorax*) and Old World screwworm (*Chrysomya bezziana)* (2019)

3.1.17. Q fever (2018)

3.1.20. Rinderpest (infection with rinderpest virus) (2018)

3.1.25. West Nile fever (2018)

Introductory note on bee diseases (2013)

3.2.5. Infestation of honey bees with *Aethina tumida* (small hive beetle) (2018)

3.2.6. Infestation of honey bees with *Tropilaelaps* spp. (2018)

3.3.1. Avian chlamydiosis (2018)

3.3.2. Avian infectious bronchitis (2018)

3.3.4. Avian influenza (including infection with high pathogenicity avian influenza viruses) (2021)

3.3.8. Duck virus hepatitis (2017)

3.3.11. Fowl typhoid and Pullorum disease (2018)

3.3.12. Infectious bursal disease (Gumboro disease) (2016)

3.4.9. Enzootic bovine leukosis (2018)

3.4.11. Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis (2017)

3.4.15. Theileriosis in cattle (infection with *Theileria annulata, T. orientalis* and *T. parva*) (2018)

3.4.16. Trichomonosis (2018)

3.6.1. African horse sickness (infection with African horse sickness virus) (2019)

3.6.6. Equine infectious anaemia (2019)

3.6.7. Equine influenza (infection with equine influenza virus) (2019)

3.6.10. Equine viral arteritis (2013)

3.6.11. Glanders and melioidosis (2018)

3.8.2. Caprine arthritis/encephalitis and Maedi-visna (2017)

3.8.3. Contagious agalactia (2018)

3.8.5. Enzootic abortion of ewes (ovine chlamydiosis) (infection with *Chlamydia abortus*) (2018)

3.8.7. Ovine epididymitis (*Brucella ovis*) (2015)

3.8.11. Scrapie (2022)

3.8.12. Sheep pox and goat pox (2017) (vaccine section)

3.9.3. Classical swine fever (infection with classical swine fever virus) (2022: diagnostic tests section)

3.9.8. Swine vesicular disease (2018)

3.9.10. Transmissible gastroenteritis (2008)

3.10.9. Verocytotoxogenic *Escherichia coli* (2008)

### 5.13. Update on WOAH Standards Online Navigation Tool Project

The Commission was updated on the WOAH Standards Online Navigation Tool, which is an innovated project aimed at providing users with streamlined access and navigation of WOAH Standards.

The project will deliver three user interfaces on the WOAH Website:

- Navigation and search tool: this interface will provide a guided navigation experience that will allow users to navigate through the WOAH Codes and Manuals.
- Recommendations for safe international trade by commodity: this interface will enable users to easily visualise recommendations for safe international trade by commodity through a comprehensive filtering system.
- Management of Standards; this interface will enable WOAH staff to efficiently manage and update WOAH International Standards, following adoption of new or revised text at the WOAH General Assembly.

The tool will be demonstrated at a kiosk at the 91st General Session in May 2024 and is projected to go 'live' in July 2024.

This project represents a significant milestone in WOAH's commitment to enhance access and utilisation of WOAH standards and contributes to the objectives of the 7th Strategic Plan to implement digital transformation, respond to Members' needs and improve WOAHs efficiency and agility.

## 6. WOAH Reference Centres

### 6.1. Update on the system for evaluating the annual reports

During the last meeting of the Commission in September 2023, a risk-based approach was introduced to the system for evaluating the annual reports to increase its efficiency while reducing workload for the Commission. This system is a semi-automated method aimed at creating an effective means of performance assessment, capable of detecting underperforming Reference Laboratories (RL) with high sensitivity. The goal was to create a system that can digitally and automatically evaluate reports.

The system employs a risk-based methodology for the initial analysis of annual reports, categorising RL as either low risk or high risk for underperformance. This categorisation is based on risk criteria identified during the Commission's September 2023 meeting, such as negative responses to identified 'Essential' questions (questions 1, 18, 19, 20, and 27 of the report template), being a new RL, scoring below 50% on average across all questions. This strategy guarantees uniform screening of all reports, flagging those requiring more thorough individual assessment by Commission members, with a focus on potential underperformers. This reduces the number of reports each BSC member needs to evaluate, thereby optimising their efforts.

Regarding questions 25 and 27, the Commission agreed that the requirement to organise or participate in inter-laboratory proficiency tests could be satisfied if the reply to either question is yes, i.e. if the proficiency tests are either with WOAH Reference Laboratories or with non-WOAH laboratories.

The Commission agreed to implement this system for the first time in the review of the 2022 reports. The Secretariat distributed the reports by identified the system equally among Commission members, reducing the number from approximatively 40 annual reports per BSC member to between 20 and 23 reports, approximately half of the previous

number. An extraordinary meeting was convened in November 2023 to finalise the evaluations of the 2022 annual reports, assess the performance of the new system, and communicate the findings within the network.

After this initial application of the system, 130 annual reports were flagged based on various risk criteria: 90 for essential issues, 12 for underperformance, eight as new laboratories, and 13 were randomly selected. A detailed evaluation of these reports revealed that 49 out of the 90 Laboratories with an essential issue were confirmed as problematic and received notification letters. Of the 12 labs scoring below 50% on average across all questions, four were contacted.

The Commission agreed that the system effectively minimised its workload and strategically targeted its efforts towards the RL that most require attention. The Commission also agreed that the system demonstrated a high sensitivity in identifying annual reports with significant risks of underperformance. However, there was a discussion regarding specific situations where laboratories are engaged with diseases having low epidemiological incidence, work with eradicated diseases, or unique disease-specific laboratories. In such cases, these laboratories may struggle to fulfil all the terms of reference and hence should be accorded special consideration. Moreover, the Commission recognises the need to standardise the criteria for issuing underperformance notification letters. Moving forward, the Commission is committed to continually testing and improving the system.

### 6.2. Applications for WOAH Reference Centre status

The Commission recommended acceptance of the following applications for WOAH Reference Centre status:

WOAH *Reference Laboratory for sheep pox and goat pox*
Sciensano, Groeselenberg, 99 1180 Uccle
BELGIUM
Tel.: + 32-2 379.05.14 / 379.06.27
E mail: nick.deregge@sciensano.be
Website: https://www.sciensano.be/en https://www.eurl-capripox.be/homepage
Designated expert: Dr Nick De Regge

WOAH *Reference Laboratory for rabies*
Veterinary Research Institute, Ministry of Agriculture
No.376, Zhongzheng Rd., Tamsui Dist., New Taipei City 251018
CHINESE TAIPEI
Tel.: +886-2 26.21.21.11 Annex 602
E-mail: aphsu@mail.nvri.gov.tw
Website: https://eng.nvri.gov.tw
Designated expert: Dr Ai-Ping Hsu

WOAH *Reference Laboratory for leptospirosis*
ICAR-National Institute of Veterinary Epidemiology and Disease Informatics (ICAR-NIVEDI),
Post Box No. 6450, Yelahanka, Bengaluru 560064, Karnataka
INDIA
Tel.: +91-80 23.09.31.36 / 31.00
E mail: b.vinayagamurthy@icar.gov.in; director.nivedi@icar.gov.in;
Website: https://www.nivedi.res.in/
Designated expert: Dr Vinayagamurthy Balamurugan

WOAH *Reference Laboratory for peste des petits ruminants*
ICAR-National Institute of Veterinary Epidemiology and Disease Informatics (ICAR-NIVEDI),
Post Box No. 6450, Yelahanka, Bengaluru- 560064, Karnataka
INDIA
Tel.: +91-80 23.09.31.36 /31.00
E mail: b.vinayagamurthy@icar.gov.in; director.nivedi@icar.gov.in;
Website: https://www.nivedi.res.in
Designated expert: Dr Vinayagamurthy Balamurugan

WOAH *Reference Laboratory for salmonellosis*
Central Veterinary Laboratory,
Ministry of Agriculture, Water and Land Reform
24 Goethe Street, P-Bag 13187, Windhoek
NAMIBIA
Tel.: +264-61 23.76.84
E-mail: Siegfried.Khaiseb@mawlr.gov.na

Designated expert: Dr Siegfried Khaiseb

WOAH *Collaborating Centre* for *Field Epidemiology*
Centre National de Veille Zoosanitaire (CNVZ)
38, Avenue Charles Nicolle, Cite Mahrajène, 1082 Tunis
TUNISIA
Tel.: (+216) 71849790 - (+216) 71849812
E-mail: kalthoum802008@yahoo.fr; baccar.vet@gmail.com;
Website: www.cnvz.agrinet.tn
Contact point: Dr Sana Kalthoum

An application had been received from a county in Africa for a Reference Laboratory for avian Influenza. The Commission was fully satisfied with the quality and capacity of the applicant institution and the services it could provide to WOAH Members. However, the Commission questioned the choice of designated expert. The Commission would seek clarification of the nominee's experience in diagnosis and research, and role in the laboratory. The applicant will be asked to provide more detailed information on their experience in standardisation and validation of diagnostic tests, as well as peer-reviewed publications on avian influenza. Although the laboratory clearly had a great deal of experience with the disease, the proposed expert did not fulfil the expectations of a WOAH Expert. The Commission therefore did not accept the application at this time.

Another application had been received from a country in the Asia-Pacific region for a Reference Laboratory for FMD. Some years ago, the Commission was made aware of a number of quality and safety issues in this laboratory. Three areas of concern were identified: the level of expertise of the designated expert; lack of trust in the choice and efficacy of the tests undertaken by the laboratory and in the safety of the reagents it produces and supplies to other laboratories; and concern about the inadequate biosafety level. The laboratory withdrew from the list of WOAH Reference Laboratories while it underwent a performance monitoring scheme (PMS) with other independent WOAH Reference Laboratories to address these issues. The Commission questioned the timing of the application as the PMS is not complete and the laboratory remains under construction. The Commission also had questions about the biosafety level at which the laboratory is currently operating, given the nature of the work being undertaken there. The Commission noted gaps in the information provided. On a positive note, the proposed designated expert submitted an excellent curriculum vitae and meets the expectations of a WOAH expert. Overall, however, the Commission found that it is premature to apply for Reference Laboratory status and did not accept the application.

Another application had been received from a country in the Asia-Pacific region for a Reference Laboratory for equine piroplasmosis. The Commission was fully satisfied with the excellence of the centre for equine diseases, notably acknowledging the applicant institution's scientific excellence and its potential to significantly contribute to WOAH as well as the expertise of the designated expert. Despite these strengths, the Commission has two major concerns: the limited range of diagnostic methods routinely employed and the laboratory's international outreach, for example the organisation and participation in international proficiency tests. The Commission did not accept the application at this time, but would encourage the applicant to address these important issues. The Commission will closely evaluate any supplementary information submitted.

Finally, an application had been received for a Collaborating Centre for Reference Materials for Molecular Diagnostic Techniques in Aquatic and Terrestrial Animal Diseases. The Commission was satisfied with the scientific excellence of the expert and believed the Centre would be a useful addition to the WOAH network. As the application was more focused on aquatic animal diseases, the Biological Standards Commission agreed that responsibility for the final decision on endorsing the application lies with the Commission on Aquatic Animals (see item 13.1 of the report of the meeting of February 2024 meeting of the Aquatic Animals Commission.

### 6.3. Changes of experts at WOAH Reference Centres

The Delegates of the Members concerned had submitted to WOAH the following nominations for changes of expert at WOAH Reference Laboratories. The Commission recommended their acceptance:

*Brucellosis* (*Brucella abortus, B. melitensis, B. suis*):
Dr Liangquan Zhu to replace Prof. Jiabo Ding at the China Institute of Veterinary Drug Control (IVDC), CHINA (PEOPLE'S REP. OF)

*Infectious bursal disease* (*Gumboro disease*):
Dr Yulong Gao to replace Dr Xiaomei Wang at the Division of Avian Immunosuppressive Disease, Harbin Veterinary Research Institute (HVRI), Chinese Academy of Agricultural Sciences (CAAS), CHINA (PEOPLE'S REP. OF)

*Sheep pox and goat pox:*
Dr Mohammand Hassan Ebrahimi-jam to replace Dr Hamid Reza Varshovi at the RAZI Vaccine & Serum Research Institute, IRAN

*Swine influenza:*
Dr Junki Mine to replace Dr Takehiko Saito at the Viral Disease and Epidemiology Research Division, National Institute of Animal Health, National Agriculture and Food Research Organization, JAPAN

*Avian influenza*:
Dr Eun Kyoung Lee to replace Dr Youn-Jeong Lee at the Animal and Plant Quarantine Agency Ministry of Agriculture, Forest and Rural Affairs, KOREA (REP. OF)

*Rabies*:
Dr Juan Antonio Montaño Hirose to replace Dr José Álvaro Aguilar Setién at the National Centre for Animal Health Diagnostic Services, MEXICO

*Leptospirosis:*
Dr Paula Ristow to replace Dr Marga Goris at the Academic Medical Centre, Department of Medical Microbiology and Infection Prevention University of Amsterdam, NETHERLANDS

*Q fever*:
Dr Agnieszka Jodelko to replace Dr Krzysztof Niemczuk at the National Veterinary Research Institute, Department of Cattle and Sheep Diseases, POLAND

*Lumpy skin disease:*
Dr Antoinette Van Schalkwyk to replace Dr David Wallace at the Onderstepoort Veterinary Institute, SOUTH AFRICA

The Commission reviewed one additional nomination for change of expert and based on the information provided found that the nominee did not fulfil the expectations of a WOAH Expert. The Member would be asked to either resubmit a strengthened curriculum vitae or to propose a different expert.

### 6.4. Review of new and pending applications for laboratory twinning

As of February 2024, 90 projects have been completed and 16 projects are underway. Of the completed projects, 15 Reference Laboratories and four Collaborating Centres have achieved WOAH designation status.

Six Laboratory Twinning project proposals were presented for the Commission's review:

1. **Jordan – United Kingdom** for foot and mouth disease: the Commission supported the technical contents of this project proposal.
2. **South Africa – Türkiye** for Rift Valley fever: the Commission supported the technical contents of this project proposal.
3. **United States of America – Romania** for Biorisk management: the Commission supported the technical contents of this proposal.
4. **Germany and Cameroon** for Newcastle disease: the Commission supported the technical contents of this proposal.
5. **United States of America – Vietnam** for rabies: the Commission supported the technical contents of this proposal.
6. **South Africa – Tanzania** for capacity development for standard diagnostic methods for small ruminant diseased: the Commission supported the technical contents of this proposal with some amendments.

### 6.5. Feedback from Laboratories that are not complying with the key ToR

The Commission reviewed the feedback received from 28 Reference Laboratories that were not complying with key performance criteria according to their 2022 annual reports. The majority of these RLs responded with acceptable justifications for their non-compliance with the ToR, and the Commission accepted their explanations. However, while the responses were accepted, all the RL will be placed on a watch list. This implies that their annual report will undergo a more thorough review during the next round of assessments to ensure compliance and progress.

Some RLs reported that they did not receive any requests for diagnostic testing because they are located in regions free from the disease. The Commission will consider how to evaluate laboratories in situations where the disease is well controlled or not widely distributed. Similarly, being the only RL for a specific disease meant that certain laboratories were unable to join or form networks, which was noted by the Commission. However, in these cases, the ToR indicate that networks can also be formed with institutions that are not WOAH RLs, and the Commission encourages RLs to establish such networks.

### 6.6. Review the template for the curriculum vitae for nominations of replacement experts

While reviewing nominations for replacement experts, the Commission identified a recurring issue with the information provided, which often is incomplete or fails to meet the required evaluation standards. To promote consistency in nominations and prevent delays caused by inadequate curriculum vitae (CV) details, the Commission agreed to review the CV template for new applicants and nominations of replacement experts at Reference Centres.

First, the Commission added more mandatory fields for basic information such as an email address and the name of the disease. To better assess their suitability, nominees, would be asked to provide a more comprehensive list of academic and professional qualifications, including the year each degree was obtained. In a separate section, experts are asked to provide information on past roles, durations, and responsibilities.

Given the need to determine the level of their expertise, nominees are now prompted to provide details of their international recognition and standing, including appointments, awards, memberships, participation in working groups and relevant activities. Finally, the section on peer-reviewed publications has been amended to ensure relevance to the field, requiring the experts to highlight their name in bold in the title of the publication, along with the disease or pathogen in question. Publications should be listed chronologically and should demonstrate the expert's ongoing contribution and current standing in their field of expertise.

The template was also approved by the Aquatic Animals Commission (see item 3.1 of the report of the February 2024 meeting of the Aquatic Animals Commission.

The amended template can be found at Annex 17 for information.

### 6.7. Feedback from Centres that are not complying with the key ToR

The Commission reviewed feedback from seven Collaborating Centres that were not meeting key performance criteria according to their 2022 annual reports. Two reasons for not complying with the key ToR were commonly cited. 1) Collaboration or activities with other Centres does not usually occur annually. The Commission understands and accepts that efforts and resources might be directed towards these activities biennially. 2) The impact of SARS-CoV-2: the Commission accepted this response for the 2022 reports, but emphasised that it does not expect the Covid-19 pandemic to be among the reasons for not complying the ToR in the 2023 annual reports.

The Commission accepted the proposals offered by the seven Centres for improving performance and placed them on a watch list for a follow-up review during the next annual report review cycle.

### 6.8. Review the proposed procedure on how to evaluate Centres at the end of their 5-year mandate

Collaborating Centres are designated for a period of 5 years, during which they adhere to a 5-year work plan, which was submitted at the beginning of the designation period. At the end of this period, the Director General sends a letter requesting a report of the achievements of the 5 years as outlined in the work plan. The Commission evaluates this report and decides if the Collaborating Centre's designation should be renewed or not based on their performance and the need to maintain a Centre for the specific topic.

This system of designating Collaborating Centres for a 5-year period was introduced in 2020 with the adoption of the SOPs (Collaborating Centre - Procedures for Designation). The first Centres to reach the end of their 5-year designation will do so at the end of 2024.

At its September 2023 meeting, the Commission agreed that a letter requesting a final report of their activities over the past 5 years in relation to the 5-year work plan originally submitted should be sent at the end of the second quarter of the fifth year of the designation. The Centres will also be requested to submit the regular annual report, and both will be assessed by the Commission.

The Commission reviewed and updated the template for this final report, including specific performance criteria. The template is designed to capture comprehensive evidence of the Centre's impacts and achievements over the 5-year period, as well as the benefits provided to the territory, region or even globally. The revised template is tailored to

gather the necessary information to assess the Centre's adherence to their planned 5-year work plan. It includes sections for detailing the goals and objectives from the original submission, indicating their current status as 'achieved', 'in progress', 'modified', or 'not started', always providing reasons for each response. Additionally, the template includes a table for summarising completed activities, with a focus on the expected and achieved benefits. To conclude, a 'Renewal' section has been added, where Collaborating Centres can express their interest in being considered for renewal. The Centre will be asked to outline their strategy for contributing to the WOAH mandate and enhancing the visibility of the Centres. They are also be asked to describe in bullet points how they can assist WOAH Members. Finally, the Commission will evaluate the relevance of the domain of activity of each Collaborating Centre in line with the WOAH Strategic Plan.

The Commission will conduct a preliminary review of these final reports, with initial results to be announced at the subsequent February meeting. Centres with approved final reports and with a clear vision to contribute to WOAH's Strategic Plan will be informed following the February meetings of the Commission of their eligibility for renewal and will be invited to present a new 5-year plan. Centres whose performance is deemed unsatisfactory or those that do not submit a report will be granted a 6-month appeal period, leading up to the next Commission meeting in September. During this meeting, their designation status will be re-evaluated, which may result in their removal from the list.

### 6.9. Review ways to improve the output of Collaborating Centres for the benefit of WOAH and Members

The Commission discussed ways of enhancing the outputs of Collaborating Centres for the benefit of the Centres themselves, WOAH and its Members. One possibility considered was to review the ToR to ensure they remain relevant and effective. Recognising the broad scope of topics covered by the Centres as a valuable resource, the Commission wondered if the current network adequately covers all the needs of Members and WOAH. The Commission agreed to focus on evaluating potential gaps between the existing areas of expertise, particularly in relation to maintaining expertise amid ever evolving technology. A significant point of discussion was how to enable Members to better leverage this resource, which might be achieved by increasing communication with Members and facilitating more effective use of the Centres. To increase their visibility, the Commission proposed to ask the Centres to submit three to five bullet points on the services they offer, which will be added to the Centre's entry on the WOAH website through a link entitled 'How can we help you'. Finally, the Commission pointed out that an important criterion for support involves maintaining contact with Reference Laboratories, ensuring a collaborative and informed network.

### 6.10. Update on the three Reference Laboratory network (ASF, PPR[5] and rabies)

**African swine fever**

The WOAH ASF Reference Laboratory network held regular virtual meetings to exchange scientific and technical expertise, including recent developments on ASF vaccines, and discussed activities in developing training programmes to assist at-risk countries, including the organisation of proficiency tests.

The network is finalising a laboratory manual, including diagnostic algorithms to detect low virulent and novel emergent ASFV variants, to explore user requirements on an open-access information sharing platform for ASF virus genome sequence data and detecting circulant recombinant virus.

**Peste des petits ruminants**

The WOAH PPR Reference Laboratory network continues to regularly update its website and organise activities in support of its members. In November 2023, the PPR Global Research and Expert Network's sixth meeting was held in Bengaluru, India, focusing on PRR research innovations to support the PPR Blueprint's second and third eradication phases. Also in November 2023 in the African region, a key cross-border harmonisation workshop and Regional Advisory Group meeting for PPR eradication took place in Grand Bassam, Cote d'Ivoire. This workshop focused on collaborative strategies for PPR risk management and eradication efforts. In December 2023, the WOAH PPR Reference Laboratory network conducted a workshop focusing on critical aspects of PPR management.

The ongoing development of the PPR Monitoring and Assessment Tool (PMAT) training e-modules is being managed by the FAO[6] Virtual Learning Centre. In parallel, the digitalisation of PMAT is progressing, marking significant strides in modernising these tools. The newly developed PPR Episystem Guidelines were presented during a virtual stakeholder meeting. Final approval is expected shortly. Finally, a revised template for developing National Strategic Plans (NSP) for PPR was crafted and presented to countries and stakeholders for adoption. This updated template is now set to be used by countries to align their NSPs with the PPR blueprints, ensuring a more cohesive and effective approach to PPR management and eradication efforts.

5 PPR: Peste des petits ruminants
6 FAO: Food and Agriculture Organization of the United Nations

**Rabies**

The WOAH Reference Laboratory Network for Rabies (RABLAB) continued to meet bimonthly to share information and align activities to improve global support for rabies diagnostics, surveillance, capacity building and implementation of rabies control activities. A second in-person meeting of the network was held 8 November 2023, in Rome, Italy, to review progress and identify key priorities for 2024.

Efforts continue to improve promotion and transparency of RABLAB activities, including an upcoming annual newsletter showing key outputs and updates from the network. BSC noted again the need to better highlight RABLAB activities on the WOAH website.

RABLAB continues to support WOAH Members through several Twinning projects to build laboratory capacity for rabies diagnosis and will support the United Against Rabies Forum in implementing the initial three pilots of the Country Partnership Programme, which aims to provide broader, One Health support for rabies-endemic countries. RABLAB experts have also contributed to the development of the 'Oral vaccination of dogs against rabies: Recommendations for field application and integration into dog rabies control programmes'.

RABLAB are continuing discussions with relevant manufacturers to explore how protocols for lateral flow devices (LFDs) can be improved to support rabies surveillance. At present, the RABLAB statement on the use of LFDs remains unchanged.

In 2024 RABLAB will continue to provide direct support rabies-endemic countries in the drafting and implementation of their National Strategic Plans, and, when appropriate, help them apply for WOAH endorsement; support WOAH in monitoring international standards to ensure these remain fit for purpose; enhance collaboration among RABLAB members; and disseminate scientific information among WOAH Members and the wider rabies community.

### 6.11. Annual reporting system for WOAH Collaborating Centres and Reference Laboratories

In December 2022, an electronic system was launched to collect annual reports from WOAH Reference Centres. Regrettably, several Reference Centres encountered difficulties in completing and submitting their reports due to bugs in the system.

To address the identified issues and enhance user-friendliness, a service provider was hired by WOAH in November 2023 to upgrade and evolve the current system based on the problems identified during its initial use. This system renovation aims to enhance and develop additional functionalities for the existing WOAH Reference Laboratories and Collaborating Centres (RL&CC) information system. The RL&CC information system must effectively collect, store, process, and submit reports of WOAH RL and CC activities, supporting decision-making, coordination, control, analysis, and visualisation of the final reports. It is designed to automate and streamline business processes, thereby reducing manual effort, mitigating potential risks, and improving operational efficiency for both WOAH and the network of CC and RL.

The system evolution will implement the use of one email address for access to both CC and RL templates facilitating those involved with both. This enhancement will allow RL and CC users to access multiple reports without the need to manually log in and out when switching reports. Furthermore, it will enable RL and CC to add multiple users to fill out and edit reports simultaneously. Additionally, the system will improve existing functionalities such as User Experience (UX) design, modify existing form templates for both RL and CC, and fix existing bugs within the system.

The current project plan envisages launching the new system in March 2024. The Commission emphasised that should the system not meet the requisite high standards in the projected timeline, its deployment will have to be postponed until it fully satisfies all the necessary quality criteria.

The Commission expressed concern about ensuring the system reaches the Organisation's level of excellence and that the Reference Centres can use a system that meets their needs. The Commission thanked the Reference Centres for their understanding regarding the postponement of annual report submissions and emphasised that their annual reports would be evaluated in September 2024.

### 6.12. Fraudulent use of the WOAH emblem/logo

The Commission was made aware of a WOAH Reference Laboratory that is using the WOAH emblem on vaccines that it is selling to Members. This is fraudulent use of the WOAH emblem/logo, which is clearly described as such in the Guidelines on the Use of the WOAH Reference Centre Emblem. The WOAH is pursuing this issue with the institution involved, which has withdrawn the products from the market. Reference Centres are reminded that to follow the Guidelines or to ask WOAH Headquarters if they have a question about how they can use the WOAH Emblem.

## 7. *Ad hoc* Groups: Update on activities of past *ad hoc* Groups

### 7.1. *Ad hoc* Group on Replacement of the International Standard Bovine Tuberculin (ISBT) and Avian Tuberculin (ISAT)

The Commission was informed that the third trial has been completed in October 2023 and based on the results, the *ad hoc* Group recommended to continue performing one last set of trials on candidate B by fine-tuning the parameters of the experiment. The *ad hoc* Group also discussed the results of all the sets of trials, which had indicated that two of the four assays were invalid. One assay however, had approached acceptability based on EU Pharmacopoeia criteria suggesting potency between 50% and 200%, and the inhouse standard provided by the manufacturer was close to the acceptable range of potency of 30,000 units. The *ad hoc* Group recommended to review the original data from the manufacturer to better understand the factors contributing to lower potency estimates. In the last set of trials, the duration of infection and the inoculation dose was increased sequentially to minimise any variables. This fourth and final trial is currently ongoing, and results are anticipated in mid-March 2024.

If the trials are favourable, the Commission will consult remotely to decide whether to identify candidate B as a replacement for ISBT at the next General Session. Furthermore, the Commission recommended that in case the trials are unfavourable, WOAH should continue to identify a new candidate and restart the trials. The Commission recommends that WOAH continue to mobilise resources to identify funding to sustain the project, as without a universally accepted standard, Members would have to rely on the manufacturer's standard, which may lead to variability in the results.

Regarding avian tuberculin, the Commission was informed of a call for donations of a candidate avian tuberculin was launched in December 2023. The last date for receiving applications from the manufacturers was 16 February 2024.the Commission recommended that the *ad hoc* Group review and recommend the shortlisted candidates to the Commission.

### 7.2. *Ad hoc* Group to Review *Terrestrial Code* Chapter 4.7. 'Collection and processing of bovine, small ruminant and porcine semen'

The Commission was informed that an expert consultation would be held virtually to develop an action plan for the work of this *ad hoc* Group. A member of the Biological Standards Commission was identified to participate in meetings of the Group.

### 7.3. *Ad hoc* Group on Emerging Diseases (including Re-Emerging Diseases) and Drivers of Disease Emergence in Animals

The Commission was informed of the activities of this Group and noted the relevant recommendations.

## 8. International Standardisation/Harmonisation

### 8.1. WOAH Register of diagnostic kits – update and review of new or renewed applications

The Secretariat for Registration of Diagnostic Kits (SRDK) informed the Commission of the status of ongoing applications. At present, there are 16 diagnostic test kits in the WOAH Register of Diagnostic Kits.

#### 8.1.1. Addition of a new diagnostic kit to WOAH's register: Genelix™ ASFV Real-time PCR Detection kit

The assessment of the application for GenelixTM ASFV real-time PCR detection kit (Sanigen) is under evaluation. The review and endorsement of conclusions, recommendations in the Review Panel Final Report and Validation Studies Abstract (VAS) will be processed by written procedure. Depending on the endorsement, a Resolution is planned: to add a new diagnostic kit to WOAH's register for adoption during the 91st General Session in 2024.

The intended purpose of the kit: the Genelix™ ASFV Real-time PCR Detection kit is a product that qualitatively detects and confirms the diagnosis of ASFV using a real-time PCR detection system in the whole blood, serum, and tissues of swine suspected of being infected with the ASFV.

The Validation Studies Abstract – Supplementary Data, drafted by the manufacturer and approved by the Expert Review Panel, was endorsed by the Commission (see Annex 18).

#### 8.1.2. Addition of a new diagnostic kit to WOAH's register: Sentinel® ASFV Antibody Rapid Test

The Commission was informed that the evaluation of the dossier on Sentinel® ASFV Antibody Rapid Test (Manufacturer: Excelsior Bio-System Incorporation) has been completed. Based on the final report from the Expert Review Panel, the Commission endorsed the Panel's recommendation to approve the kit's 'fitness for purpose' as described in the Validation Studies Abstract and User's Manual (Instructions for Users).

The Sentinel® ASFV Antibody Rapid Test is an immuno-chromatographic lateral flow assay (LFA) intended for the detection of ASFV antibodies in porcine serum samples. The test is designed to be used for the diagnosis of ASFV infection, in conjunction with other tests or diagnostic procedures, and the evaluation of antibody response to infection.

The Validation Studies Abstract drafted by the manufacturer and approved by the Expert Review Panel was endorsed by the Commission (see Annex 19).

A Resolution will be prepared accordingly to add a new diagnostic kit to WOAH's register for adoption during the 91st General Session in 2024.

#### 8.1.3. Decision of the 5 year-Renewal and a Resolution's: Avian Influenza Antibody test kit (registration number 20080203) BioChek (UK) Ltd

The Commission endorsed the recommendation for the 5-year renewal with a Resolution for the Avian Influenza Antibody test kit (registration number 20080203) BioChek (UK) Ltd, based on the provided information and in accordance with the agreed procedure.

#### 8.1.4. Decision of the 5 year-Renewal and a Resolution's: Newcastle Disease Antibody test kit (CK116; registration number 20140109) BioChek (UK) Ltd

The Commission endorsed the recommendation for the 5-year renewal with a Resolution for Newcastle Disease Antibody test kit (registration number 20140109) BioChek (UK) Ltd based on the provided information and in accordance with the agreed procedure.

#### 8.1.5. Update on the WOAH Register of diagnostic kits

Following the information given to the Commission in February 2023 (agenda item **8.1.7.**) about the Future Secretariat for Registration of Diagnostics Kits (SRDK), the Commission was informed that, in agreement with the Director General and Deputy Director General, International Standards and Science, SRDK will proceed with the complete freeze of the Diagnostic Kits Register's activities and all related procedures starting after the 91st General Session, for a renewed period of 24 more months, thus, until May 2026. This will mean:

- Validated and approved kits will maintain their certification;
- No renewal processing, even if they arrive to the 5-year due date;
- Withdrawal of all incomplete applications, with return of fees to applicants;
- No review of any potential appeal procedure;
- No review or validation of new applications;

- Consideration of exceptional cases, linked to an emergency animal health situation, upon Members request.

### 8.2. Standardisation programme

#### 8.2.1. Project to extend the list of WOAH-approved reference reagents: review of guidelines

At the last meeting in September 2023, the Commission decided to send the current guidelines (for antibody standards[7], antigen standards[8] and PCR assays[9]) to the disease-specific networks, namely ASF, FMD, rabies and PPR, with the request that they establish minimum criteria for the development of reference reagents so that the guidelines could be made more achievable while maintaining the quality of the reagents

7 https://www.woah.org/app/uploads/2021/03/a-guideline-antibody-standards.pdf
8 https://www.woah.org/app/uploads/2021/03/a-guideline-antiegen-standards.pdf
9 https://www.woah.org/app/uploads/2021/03/a-guideline-pcr-standards.pdf

produced.

The PPR network had replied to the request and submitted minimum guidelines for the preparation and validation of reference materials for PPR diagnostic methods. The Commission reviewed the guidelines, made amendments to make them generic, and proposed that they be provided to the other networks for comment and approval before they are made available on the WOAH website. It is hoped that these guidelines will encourage more laboratories to apply to have their reagents approved by WOAH as reference reagents.

#### 8.2.2. Association française de normalisation: follow-up from September 2023

Following the September 2023 meeting, the Commission deliberated on the current status of AFNOR, noting that they have an Agreement of Liaison with WOAH. The Commission noted that the current status does not clarify if AFNOR has the jurisdiction to independently comment on WOAH Standards. The established agreement allows WOAH to act as a liaison organisation, participating in the CEN/TC's activities. The Commission noted that receiving feedback under these terms might inadvertently set a precedent, allowing multiple organisations to offer comments on the Standards, potentially leading to an unsustainable situation.

In light of this, the Commission decided that a thorough examination of the liaison agreement by WOAH's Legal Affairs Unit is necessary to clarify the scope of the agreement.

Finally, the Commission reiterated its recommendation that AFNOR send its feedback through a Member, using the representation of a designated official Delegate.

## 9. Resolutions for the General Session

The Commission noted that the following resolutions would be proposed for adoption at the General Session in May 2024:

- A resolution proposing the adoption of 13 draft chapters for the *Terrestrial Manual*;

- A resolution proposing the new WOAH Collaborating Centres.

The following resolutions would be proposed for adoption by the alternative procedure developed in May 2020 in response to the Covid-19 pandemic, where Delegates submit their votes through an online mechanism available before the General Session in May 2024:

- A resolution proposing the new WOAH Reference Laboratories for terrestrial animal diseases;

- A resolution on the WOAH Register of Diagnostic Kits.

## 10. Conferences, Workshops, Meetings

### 10.1. Update on the WOAH seminar to be held during the WAVLD Symposium in Calgary, Canada in 2025

The World Association of Veterinary Laboratory Diagnosticians has a mission to improve animal health, human health, and One Health by facilitating the availability of quality laboratory testing provided by veterinary diagnostic laboratories around the world. As part of their mission, they hold an international symposium every two year. This symposium brings together veterinary diagnosticians and others involved in veterinary laboratory diagnostics. The next ISWAVLD will be held in Calgary, Canada from 12 to 14 June 2025 and will adopt the One Health theme of 'Partnerships in Health: from Disease Detection to Prevention' with a focus on one health, antimicrobial resistance, disease detection, and outbreak response bringing veterinary medicine, human medicine, and industry together.

Traditionally, the Biological Standards Commission organises in parallel a 1-day seminar during the Symposium that will be held on 13 June 2025. The Commission discussed various topics that could be of interest for the next Seminar and suggested to invite presentations from WOAH disease-specific networks on ASF, PPR, Rabies, FMD and Avian influenza on the latest technologies for disease diagnosis, case studies of recent communicable disease such as Japanese encephalitis spread in Australia, the emergence of Western equine encephalitis in South America, a summary on the pros and cons of POCTs and how to integrate them in field diagnosis, information on validation techniques, whole genome sequencing and metagenomics, artificial intelligence, bioinformatics, impact of the Nagoya Protocol in animal health, etc. The Secretariat will contact various speakers on the suggested topics to draft a provisional agenda for discussion in the September meeting.

### 10.2. Vaccination and Surveillance for HPAI in poultry: Current situation and future perspectives

A workshop entitled 'Vaccination and Surveillance for HPAI in poultry: Current situation and perspectives' organised by IABS (International Alliance for Biological Standardization) in partnership with WOAH will be held at WOAH Headquarters from 22 to 23 October 2024. The aim of the workshop is to discuss how to implement surveillance in vaccinated poultry populations along with other aspects of HPAI vaccination. Participation is expected by a wide variety of stakeholders including Delegates, scientists, international organisations, poultry breeding and biological companies, animal welfare organisations, human health. Recommendations will be prepared and presented by a designated panel.

The organisation will waive the registration fees to WOAH Delegates and designated WOAH reference laboratory experts.

## 11. Matters of interest for consideration or information

### 11.1. Update on OFFLU[10]

The Commission was briefed on OFFLU and WOAH activities on avian influenza. During the reporting period, the avian influenza epidemic continued with high numbers of detections reported globally in poultry and non-poultry including wild birds and the first incursion of the HPAI H5 virus in the Sub-Antarctic region was detected in October 2023 in South Georgia Island. OFFLU experts point out that the negative impact of HPAI H5 on Antarctic wildlife could be immense and can result in high mortality.

In December 2023, WOAH published a policy brief on the use of avian influenza vaccination: 'Avian influenza vaccination: Why it should not be a barrier to safe trade'. The purpose of this document is to remind national authorities that vaccination, when used in accordance with WOAH international standards, is compatible with safe trade in domestic birds and their products.

For the September 2023 WHO vaccine composition meeting, data for 1368 HPAI H5 and 117 H9 avian influenza genetic sequences were contributed by animal health laboratories in countries representing Africa, the Americas, Asia, Europe and Oceania. Additionally, data for 191 swine H1 sequences and 49 swine H3 sequences were analysed and submitted. Antigenic characterisations were undertaken by OFFLU contributing laboratories and subsequently there were updates to the WHO recommendations for the development of new candidate vaccine viruses for pandemic preparedness purposes.

OFFLU embarked on a project called avian influenza matching (AIM) to provide real time antigenic characteristics of circulating avian influenza viruses in different regions to support poultry vaccination. A preliminary pilot project has been taking place involving selected Reference Centres and OFFLU experts. In October 2023, the report was released presenting the results of this project to support stakeholders and countries in their decisions regarding vaccine selection and vaccine match.

The Biological Standards Commission, with the support of WOAH Reference Laboratories avian influenza experts, are reviewing the current *Terrestrial Manual* chapter on avian influenza for an in-depth revision with the aim for adoption in May 2025.

The implementation of the resolution framework on avian influenza (June 2023–May 2025) is progressing through a dedicated monitoring & evolution tool that collects, tracks, and evaluates the execution of activities on a quarterly basis aligned with the mandate outlined in Resolution No. 28 to combat avian influenza.

The development of the new GF-TADs HPAI strategy for 2024–2033 is ongoing and the draft strategy is set to undergo consultations and commenting process with different stakeholders including Members in March 2024 aiming for a launch in May 2024.

### 11.2. Update on rinderpest

The Commission was updated on the rinderpest post-eradication activities. WOAH continues to work in partnership with FAO to reduce the RVCM[11] holdings around the world, with the exception of diagnostic materials and vaccines as part of the 'second phase' of the post-eradication era. This effort will lead to a reduction in the number of FAO-WOAH designated RHFs[12] Category A, in addition to the reduction of RVCM held by WOAH Members in unauthorised institutes.

10 OFFLU: Joint WOAH-FAO Network of Expertise on Animal Influenza
11 RVCM: Rinderpest virus-containing materials
12 RHF: Rinderpest holding facilities

Unfortunately, there has not been any progress in the sequestration or destruction of RVCM in the five Members that hold these materials outside of FAO-WOAH designated RHFs, despite several in-person and virtual discussions having been held. With regards to preparedness, the Ethiopia National Veterinary Institute has been given exceptional permission to produce two million doses of RBOK vaccine to replenish the reserve at AU-PANVAC[13] after a thorough inspection and review of procedures. WOAH hosted a meeting on 25 October 2023 to review repository inspection SOPs together with representatives of the smallpox and polio secretariats and the EuFMD[14]. The outcome of the assessments from the 2022 RHF inspections were considered, and the recommendations from the meeting will be applied in the 2024 inspections.

The biennial meeting of the FAO-WOAH designated RHF Network took place in Paris 6–7 December 2023. The members of the network updated their terms of reference and drafted a work plan for the term 2024–2026. The members of the network highlighted the need for more frequent simulation exercises to test the Global Rinderpest Action Plan and the vaccine deployment mechanism. The RHFs also encouraged FAO and WOAH to foster closer cooperation with the PPR GREN[15].

The new members of the FAO-WOAH Joint Advisory Committee (JAC) for Rinderpest were invited in January 2024. The next meeting of the JAC will be held virtually in the second quarter of 2024 and be focused on the global reduction of RVCM, advocacy with outstanding countries, and emergency preparedness.

### 11.3. Update on Global Burden of Animal Diseases programme

2024 is a transition year for WOAH's role within the GBADs Programme. The programme continues to be in a scientific discovery phase and more time is needed to establish robust and systematic analytical methods. The expertise required at this stage is being filled by academic and research institutions of the GBADs consortium. Thus, WOAH has decided to reposition the organisation's involvement in GBADs and step back from its co-leadership and lead-grantee role. WOAH should continue assuming an advisory and steering role to contribute to evaluating GBADs' scientific robustness from a fit-for-purpose for WOAH Members perspective, and advise on the programme direction to ensure consistency and usefulness for WOAH Members policy needs. This change is not immediate, as WOAH would honour its role as lead grantee to active grants for their respective lifespan until the last grant closes (in 2025). Notwithstanding, as of May 2024, WOAH would withdraw from its role as co-leader of the GBADs consortium. Once the research-centred phases are complete and the methodologies have proven utility to WOAH Memberships and national Veterinary Services, WOAH may reconsider its engagement in GBADs: This may include facilitating GBADs sustainable rollout or institutionalisation by using GBADs methodologies to inform WOAH guidelines on animal health economics, potential WOAH standards, and training materials for Members.

### 11.4. Update on DIVA vaccines for peste des petits ruminants

The current PPR live-attenuated vaccines are safe, inexpensive and effective and provide long-lasting immunity following a single immunisation. However, these vaccines have drawbacks: first, they are thermolabile and thus expensive to deliver due to the cold chain requirement, secondly, the immune response is identical to natural infection, therefore it is not possible to differentiate infected from vaccinated animals. This is an important issue because serological surveys would lead to confusion in determining whether the virus has been eliminated by vaccination.

There are several technologies developed to achieve DIVA goals noting that recombinant and vector-based vaccines expressing viral subunits can provide an alternative to conventional vaccines, as they can easily be paired with DIVA diagnostic tools. This will be useful during the eradication phase of PPR to prove that previously PPR-free, but DIVA-vaccinated susceptible animal population is free from infection by employing DIVA tests.

*Poxvirus vectored vaccines*

Capripoxvirus-vectored vaccines have also been developed against PPR that act as dual vaccine to protect against both PPR and sheep and goat pox.

The capripox vectored vaccine has been described by Fakri *et al*. (2018), has been taken up by a commercial company in Africa and was identified as a candidate for production under the market name 'Combivax POX-PPR'. The vaccine was found to be relatively thermo-stable, though it did not elicit optimum antibody response probably because of the pre-existing immunity against vector.

The progress in the registration and production of the vaccine is not available yet.

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13 AU-PANVAC: African Union Panafrican Veterinary Vaccine Centre
14 EuFMD: European Commission for the Control of Foot-and-Mouth Disease
15 PPR GREN: PPR Global Research and Expertise Network

*Adenovirus vectored vaccine*

Replication-deficient adenovirus 5 (Ad5) is considered a good recombinant vector for use in small ruminants because they lack any pre-existing immunity to this vector (Thacker *et al.,* 2009). Immunisation of goats with PPR Ad-H alone or Ad-F has been found to induce potent antibody and cell-mediated immune response though the combination of Ad-H and Ad-F induced better protection. Several reports have described Ad5 vectored PPR recombinant technology and the possibility of DIVA capabilities.

A commercial company in Africa has also identified the Adenovirus vectored PPR vaccine as a candidate for production under the market name 'Adeno-PPRH'. However, progress in registration and production is not available.

*Newcastle disease virus vectored vaccine*

Newcastle disease virus (NDV) vectored vaccine has been shown to protect against PPR and has DIVA-applicability and a high thermal tolerance.

A commercial company in Africa has also identified the Adenovirus vectored PPR vaccine as a candidate for production under the market name 'Combivax ND-PPR'. However, progress in registration and production is not available.

*Bovine herpes vectored vaccines*

Bovine herpesvirus-vectored vaccine delivering PPR virus haemagglutinin has been shown to induce both neutralising antibodies and cell-mediated responses16. The vaccine is reported as a DIVA candidate to protect against PPRV herd infection and is potentially applicable to eradication programmes.

There is no information on field trials, registration and production.

### 11.5. Update on VICH [16] activities: the 42th VICH Steering Committee and 16th Forum meeting took in Tokyo 13–16 November 2023

The Commission was informed about the 42nd VICH Steering Committee and 16th Forum meeting, which took place in Tokyo from 13 to 16 November 2023. It was highlighted that the VICH Steering Committee (SC) agreed to criteria for countries to progress along VICH membership categories as a consequence of restructuring of VICH. This effort was a continuation of work to modernise the organisation's structure and better align the VICH Forum with members' diverse expectations. In addition, Switzerland became a new observer member to VICH.

The Steering Committee also initiated two new activities related to:

(1) Global Regulatory Dossier Framework for Veterinary Medicinal Products; and
(2) Principles for technical guidance for the transition to in-vitro methods for batch potency tests of veterinary immunologicals.

The Biological Expert Working Group made progress regarding the 'Test on the Presence of Extraneous Viruses in veterinary vaccines'. The first draft of the Guideline has been prepared. The Guideline will be shared during the consultation phase with the Commission and WOAH Delegates and their respective Focal Points of Veterinary Products. The subgroup has finalised its tasks as three Guidelines (GL 50, 55 and 59) Harmonization of Criteria to Waive Animal Batch Safety Testing for implementation phase.

Training material was developed by JMAFF (Ministry of Agriculture, Forestry and Fisheries of Japan) and is available on the VICH website: https://www.vichsec.org/en/training.html on GL 50, 55 and 59).

GL 50: Harmonisation of criteria to waive target animal batch safety testing for inactivated vaccines for veterinary use.
GL 55: Harmonisation of criteria to waive target animal batch safety testing for live vaccines for veterinary use.
GL 59: Harmonisation of criteria to waive laboratory animal batch safety testing for vaccines for veterinary use https://www.vichsec.org/en/guidelines/biologicals/bio-safety/target-animal-batch-safety.html

### 11.6. Update on the virtual biobank project

The Commission was updated on the Virtual Biobank project. The project is managed by the WOAH Collaborating Centre for Veterinary Biological Biobank, hosted by the Istituto Zooprofilattico Sperimentale della Lombardia e

16 VICH is a trilateral (EU-Japan-USA) programme aimed at harmonising technical requirements for veterinary product registration. Its full title is the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products

dell'Emilia Romagna (IZSLER), Italy, and WOAH. This project consists of a web-based catalogue of biological resources held in biobanks, representing a source of information to search, locate, and retrieve samples, especially diagnostic reagents and reference reagents, along with associated metadata.

After the project's reactivation in April 2023, the Collaborating Centre held monthly meetings with WOAH for its development. The Commission was informed that since the last meeting, functionalities of the system, such as catalogue search, cart management, and request panel, have been developed. The website is currently under development to add features like access to WOAH Standards, a news section, and multilingual capabilities.

The Commission was provided with a demonstration of the latest advancements in the web platform. During this presentation, it was emphasised that the system is not designed as a direct purchasing. Instead, its primary function is to offer a biological resources catalogue and serve as a facilitative intermediary, connecting laboratories possessing biological resources with potential buyers. This approach is intended to streamline the process of accessing these resources, ensuring efficient and effective communication between laboratories and interested parties.

The Commission congratulated the Collaborating Centre on the progress and development of the project. Nonetheless, they underscored the critical need to maintain high quality standards for both the supplier laboratories and the products offered. The Commission particularly stressed the significance of adhering to ISO 17025 quality standards for this system. Moreover, concerns were raised about the ongoing maintenance of WOAH Standards and the potential implications of WOAH's responsibility in relation to these products. The question of whether to restrict this system to Reference Centres was also raised in regard to ensuring the quality of the products in the biobank. The Commission decided to table this suggestion for further evaluation during the system's next presentation.

While the Commission is interested in the correct development of this system, the Commission members want to closely monitor it to ensure that it guarantees the quality of the laboratories, their products and the maintenance of WOAH Standards, while remaining sustainable.

### 11.7. WAHIAD[17] and WAHIS[18] Platform updates

The Commission was updated on the state of play and timeline of the development and evolutions of the platform in 2023, which included the optimisation of the early warning and 6-monthly report modules, and the development of the annual report module.

The Commission was informed that sessions were organised in 2023 with members of the commissions to demonstrate how to use WAHIS functionalities and to gather feedback on their needs. Similar sessions will follow in 2024 and the Commission was encouraged to take part in them. The Commission was briefed on the relevant updates of the WAHIS Reference Tables done in December 2023. The objective of this work was to align with the changes adopted in the Terrestrial and Aquatic Animal Health Codes and Manuals at the 2023 General Session. The Commission commended this work and agreed that good communication between the Secretariat and WAHIAD regarding the work that may result in changes to the Codes/Manuals that will need to be reflected in WAHIS behaviour or functionality will allow WAHIAD to advise of any limitations or constraints that may exist from a platform reporting perspective. Finally, the Commission was informed that WAHIAD will collaborate with Standards Department. This work will allow WAHIAD to actively participate in the standard-setting process by providing inputs to the relevant Commission. This collaborative work will start with the Terrestrial Animal Health Standards Commission, but the aim is to progressively extend it to the other Commissions.

### 11.8. PVS tool

The Commission was updated on the advancements of the development of the Performance of Veterinary Services Pathway Information System (PVS IS). The PVS IS caters to the direct stakeholders of the PVS Pathway, which include Delegates and National Focal Points, institutional partners and donors, as well as PVS Experts who provide expertise and conduct PVS missions upon the request of WOAH Members. Delegates and National Focal Points will have a wealth of data at their disposal via interactive visuals and graphics showing the strengths, weaknesses, and recommendations to decision-makers for more impactful investment case development for the Veterinary Services. The PVS IS aims to meet the evolving needs of Veterinary Services, to facilitate performance improvements by offering greater insight in addition to the narrative-based PVS Reports. Offering a complete documentation of the performance of the Veterinary Services, the PVS Report contains insights unlocked by WOAH so that governments, investors, and partners can access, use, and act upon their recommendations more easily.

The innovation behind the PVS IS unlocks the power of historical data and insight contained in PVS Reports. Focusing on the strengths, weaknesses, and recommendations for each PVS Critical Competency, WOAH has migrated all

17 WAHIAD: World Animal Health Information and Analysis Department
18 WAHIS: World Animal Health Information System

essential information to its database. This allows for a quick and systematic analysis of PVS trends. For the first time, WOAH is using natural language processing and machine learning. A key result of this novel approach is greater insight into the most common and persistent recommendations, strengths and weaknesses of the Veterinary Services across the globe. Members can access this analysis via interactive dashboards with major indicators updated in real time as new data becomes available. A soft launch will progressively unveil the Information System to WOAH's network – its staff, Members, PVS experts, partners, and donors – before culminating in its global launch in May 2024.

### 11.9. Update on the Grand Challenge for sustainable laboratories

For over 10 years WOAH has been working with Global Affairs Canada, UK's International Biosecurity Programme, Chatham House, and WHO to improve sustainability of labs (particularly in low resource settings). One stream of this work programme has focused on exploring the use of open innovation to find solutions to improve laboratory sustainability. Over the past year, WOAH has led a study (subcontracted to Grand Challenges Canada) to assess the feasibility of running an open innovation initiative. The final report was delivered in July 2023.

WOAH, GAC[19], and WHO could not run an open innovation initiative successfully on their own because they need additional resources (beyond what can be offered by existing investment partners); additional expertise (fund raising, private sector engagement, innovation specialists), and representation of the development and philanthropy sectors, and in November 2023, WOAH held a meeting at Wilton Park, UK to engage key stakeholders in a consortium to take forward an open innovation initiative to improve the sustainability of diagnostic laboratories.

Forty participants had been invited including potential investment partners, technical experts (laboratories and innovation). The meeting was a success and achieved its objectives: 1. The whole group agreed that laboratory sustainability was a problem that needed to be addressed. 2. A core group of high calibre representatives (from key sectors) showed strong interest and agreed to be part of a working group to develop a work plan to take forward the initiative, the workplan would include fund raising, advocacy, technical innovation. This group included the White House/USA (Maj. Gen. Paul Friedrichs); European Commission (Anne Sophie Lequarre); African Union (Aggrey Ambali); Global Health Security Fund (Andrew Nerlinger); Effective Giving (Joshua Monrad); Gates Foundation (David Blazes); Australian Government (Phoebe Readford) plus existing leaders (WOAH, UK, Canada, WHO).

Since then, WOAH has developed an elevator pitch and the initiative has been branded as BIO-PREVAIL, which stands for Biological Preparedness and Resilience through Evolution and Innovation of Laboratories.

The informal working group formed at the Wilton Park meeting will develop a work plan and governance structure; look for opportunities for engagement and advocacy, including the possibility of a side event at UN General Assembly

### 11.10. Biosafety Research Roadmap

After meeting regularly for 2 years the WOAH Technical Working Group delivered six scientific papers to support the implementation of evidence-based laboratory biological risk management[20]. Following peer review, the papers were published as open access in Applied Biosafety. One paper provides an overview of the project, five others provide a review of the evidence base to support commonly used biosafety measures for selected pathogens (*Bacillus anthracis, Brucella melitensis*, SARS-CoV-2, Mpox virus, avian influenza, *Mycobacterium tuberculosis, Shigella* spp., FMD virus)

The project has also delivered a 20-year review of laboratory accidents and laboratory escapes in human and animal health laboratories. The study itself and a commentary paper were published in *The Lancet Microbe* in December 2023[21]. The papers call for more transparency around laboratory accidents to support biological risk management, ultimately mitigate against future accidents, and for greater investment in biosafety professionals.

A joint WOAH, WHO and Chatham House workshop also developed a paper which was aimed at high level decision makers and funders. It has been published as a Chatham House paper[22].

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19 GAC: Global Affairs Canada
20 https://www.liebertpub.com/doi/10.1089/apb.2022.0040
https://www.liebertpub.com/doi/10.1089/apb.2022.0042
https://www.liebertpub.com/doi/10.1089/apb.2022.0039
https://www.liebertpub.com/doi/10.1089/apb.2022.0045
https://www.liebertpub.com/doi/10.1089/apb.2022.0038
https://www.liebertpub.com/doi/10.1089/apb.2022.0046
21 https://www.thelancet.com/journals/lanmic/article/PIIS2666-5247(23)00288-4/fulltext
22 https://www.chathamhouse.org/laboratory-accidents-and-biocontainment-breaches/issues-need-be-addressed

The Biosafety Research Roadmap was also discussed at a side event at the 2024 Prince Mahidol Award Conference in Thailand during a panel discussion. This panel also discussed the need to manage risk all along the pathogen value chain including from sample collection all the way through to pathogen destruction or inactivation. Traditionally, biological risk management has focussed on certain critical control points along the pathogen value chain such as sample shipment or sample/pathogen manipulation in the laboratory. However, there is increasing recognition that biological risk management should be applied all along the chain. WOAH suggested that there may be gaps in WOAH standards (which focus on laboratories and shipment) and that there could be value in WOAH developing some standards to manage risks all along the pathogen value chain. The Commission agreed that this would be a good idea and that work could be initiated in this area.

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…/Annexes

### Annex 1. Adopted Agenda

**MEETING OF THE WOAH BIOLOGICAL STANDARDS COMMISSION**

**Paris, 5–9 February 2024**

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1. Welcome
   - 1.1. Director General
   - 1.2. Deputy Director General, International Standards and Science
   - 1.3. Updates from the WOAH Headquarters
2. Adoption of Agenda
3. **Collaboration with other Commissions**
   - 3.1. Horizontal issues among the Specialist Commissions
     - 3.1.1. Review of case definitions: tularemia, infection with avian metapneumovirus (turkey rhinotracheitis)
   - 3.2. Scientific Commission for Animal Diseases
     - 3.1.1. Nothing for this meeting.
   - 3.3. Terrestrial Animal Health Standards Commission
     - 3.3.1. Updates from the September 2023 Code Commission meeting
     - 3.3.2. Biological Standards Commission's recommendations to the Terrestrial Animal Health Standards Commission
     - 3.3.3. Update from the Biological Standards Commission on the request from the Code regarding *Terrestrial Code* Chapter 6.10 Responsible and prudent use of antimicrobial agents in veterinary medicine
     - 3.3.4. Question on the chapter on bovine viral diarrhoea
     - 3.3.5. Framework for Terrestrial Code standards (disease-specific chapters)
   - 3.4. Aquatic Animal Health Standards Commission
     - 3.4.1. Nothing for this meeting.
4. Work Programme
5. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals
   - 5.1. Format of the report and commenting system
   - 5.2. Review of Member comments received on draft chapters and their endorsement for circulation for second-round comment and proposal for adoption in May 2024
   - 5.3. Fast-track revision of the chapter on avian influenza: Follow-up from the Animal Health Forum and the adopted Resolution on avian influenza
   - 5.4. Update on Chapter 2.3.1 The application of biotechnology to the development of vaccines for veterinary use
   - 5.5. Update on draft chapter on diagnostic validation of point-of-care tests for WOAH-listed viral diseases using field samples
   - 5.6. Follow-up from September 2023: conclusions and recommendations from the WOAH *Scientific and Technical Review* issue on diagnostic test validation science
     - 5.6.1. Progress on development of a validation report form for tests recommended in the *Terrestrial Manual*
     - 5.6.2. Progress on development of a template for a new *Terrestrial Manual* section on the rationale behind the selection of tests included in Table 1. *Test methods available and their purpose*
   - 5.7. Application of the criteria for keeping chapters in the *Terrestrial Manual* on non-listed diseases
   - 5.8. Review of advice submitted by experts of seven *Terrestrial Manual* chapters updated and circulated in October 2023 on whether the update had an impact on the corresponding chapter in the *Terrestrial Code*
   - 5.9. Update on the request from the Code Commission regarding Chapter 2.1.1 Laboratory methodologies for bacterial antimicrobial susceptibility testing
   - 5.10. Request to reconsider inclusion of foot and mouth disease virus-like particles in the WOAH *Terrestrial Manual*
   - 5.11. Follow-up from the General Session: proposal to include a vaccine in the chapter on American foulbrooD

5.12. *Terrestrial Manual* status: update on chapters selected for the 2024/2025 review cycle

5.13. Update on WOAH Standards Online Navigation Tool Project

**6. WOAH Reference Centres**

6.1. Update on the annual reporting system

6.2. Applications for WOAH Reference Centre status

6.3. Changes of experts at WOAH Reference Centres

6.4. Review of new and pending applications for laboratory twinning

*Reference Laboratories – Implementation of the SOPs*

6.5. Feedback from Laboratories that are not complying with the key ToR

6.6. Review the template for the curriculum vitae for nominations of replacement experts

*Collaborating Centres – Implementation of the SOPs*

6.7. Feedback from Centres that are not complying with the key ToR

6.8. Review the proposed procedure of how to evaluate Centres at the end of their 5-year mandate

6.9. Review ways to improve the output of Collaborating Centres for the benefit of WOAH and Members

*Reference Centre networks*

6.10. Update on the three Reference Laboratory networks (African swine fever, peste des petits ruminants and rabies)

6.11. Collaborating Centres and Reference Laboratories reporting system

**7. *Ad hoc* Groups: Update on activities of past *ad hoc* Groups**

7.1. *Ad hoc* Group on Replacement of the International Standard Bovine and avian Tuberculin (ISBT): update on the replacement ISBT and ISAT

7.2. *Ad hoc* Group on Alternative Strategies for the Control of *Mycobacterium tuberculosis* Complex (MTBC) Infection and Bovine Tuberculosis (BTB) Disease in Livestock Species

7.3. *Ad hoc* Group to Review *Terrestrial Code* Chapter 4.7. Collection and processing of bovine, small ruminant and porcine semen

7.4. *Ad hoc* Group on Emerging Diseases

**8. International Standardisation/Harmonisation**

8.1. WOAH Register of diagnostic kits: update and review of new or renewed applications

8.1.1. Addition of a new diagnostic kit to WOAH's register: Genelix™ ASFV Real-time PCR Detection kit

8.1.2. Addition of a new diagnostic kit to WOAH's register: Sentinel® ASFV Antibody Rapid Test)

8.1.3. Decision of the 5 year-Renewal and a Resolution's: Avian Influenza Antibody test kit (registration number 20080203) BioChek (UK) Ltd

8.1.4. Decision of the 5 year-Renewal and a Resolution's: Newcastle Disease Antibody test kit (CK116; re

8.2. Standardisation programme

8.2.1. Project to extend the list of WOAH approved reference reagents: review of guidelines

8.2.2. Association française de normalisation: follow-up from September 2023

**9. Resolutions for the General Session**

**10. Conferences, Workshops, Meetings**

10.1. Update on the WOAH seminar to be held during the WAVLD Symposium in Calgary, Canada in 2025

10.2. Vaccination and Surveillance for HPAI in poultry: Current situation and future perspectives; week of 21 October 2024 at WOAH Headquarters. A 2- to 3-day meeting, organised by IABS in partnership with WOAH

**11. Matters of interest for consideration or information**

11.1. Update on OFFLU

11.2. Update on rinderpest

11.3. Update on Global Burden of Animal Diseases programme

11.4 Update on DIVA[1] vaccines for peste des petits ruminants

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1 DIVA: Detection of infection in vaccinated animals

11.5. Update on VICH activities: the 42th VICH Steering Committee and 16th Forum meeting took in Tokyo 13–16 November 2023
11.6. Update: Health for Animals
11.7. Update on the virtual biobank project
11.8 WAHIAD and WAHIS Platform updates
11.9. PVS tool
11.10. Update on the Grand Challenge for sustainable laboratories
11.11. Biosafety Research Roadmap
11.12. Update on activities under the IHSC[2]-WOAH collaboration agreement and consultancy project in Asia (Horse related matters: Consultancy projects in Asia and South America)

2 IHSC: International Horse Sports Confederation

## Annex 2. List of Participants

## MEETING OF THE BIOLOGICAL STANDARDS COMMISSION

**Paris, 5–9 February 2024**

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### MEMBERS OF THE COMMISSION

**Prof. Emmanuel Couacy-Hymann**
(President)
Professor of Virology
CNRA/LIRED
Abidjan
CÔTE D'IVOIRE

**Prof. Ann Cullinane**
(Vice-President)
Head of Virology Unit
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**Dr John Pasick**
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Formerly National Centre for
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**Dr Joseph S. O'Keefe**
(Member)
Head of Animal Health Laboratory
Ministry for Primary Industries
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**Dr Satoko Kawaji**
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**Prof. Chris Oura**
(Member)
Professor of Veterinary Virology
The University of the West Indies
St-Augustine
TRINIDAD AND TOBAGO

### CONSULTANT EDITOR OF THE *TERRESTRIAL MANUAL*

**Dr Steven Edwards**
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### WOAH HEADQUARTERS

**Dr Gregorio Torres**
Head
Science Department

**Ms Sara Linnane**
Senior Scientific Officer
Science Department

**Dr Gounalan Pavade**
Senior Scientific Coordinator
Science Department

**Dr Charmaine Chng**
Deputy Head
Science Department

**Dr Mariana Delgado**
Scientific Secretariat Officer
Science Department

**Annex 3. Work Programme for the WOAH Biological Standards Commission**

**MEETING OF THE BIOLOGICAL STANDARDS COMMISSION**

**Paris, 5–9 February 2024**

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| Subject | Issue | Status and Action |
|---|---|---|
| **Updating the *Terrestrial Manual*** | 1) Circulate the chapters approved by the BSC to Members for second-round comment and proposal for adoption in May 2024 | March 2024 |
| | 2) Remind authors of the chapters identified previously for update but not yet received and invite authors of chapters newly identified for update | On-going |
| | 3) Upload, publicise and inform Reference Laboratories experts about the database of validation reports to be published on the WOAH Website for tests recommended in the *Terrestrial Manual* | On-going |
| | 4) Include as appendices at the end of the disease-specific chapters, the tables explaining the scores given to the tests in Table 1 *Test methods available and their purpose*. Add links to the validation reports when available (point 3 above). | Accomplished |
| | 5) Ask Reference Centres to provide links to suitable instructional videos to be added to the end of the disease-specific chapters. Videos to be reviewed by the Commission when the chapter is up for review | On-going |
| | 6) Develop criteria for removing chapters for non-listed diseases and assess those chapters against the criteria | Accomplished |
| | 7) Review new developments in diseases causing significant global impacts (e.g. avian influenza, African swine fever) and prioritise those chapters | On-going |
| | 8) Start the process of addressing the request to have access to the previous versions and evolution of the *Terrestrial Manual* as done with the *Terrestrial Code* | On-going |
| **Collaborating Centres** | 1) Implementation of the adopted SOPs: | |
| | a) Develop a template for the Collaborating Centres for the report of their assessment of their performance in the past 5-years to be compared with their initial 5-year work plan | Accomplished |
| | b) Send the 5-year working plan evaluation template to the appropriate Collaborating Centres | July 2024 |
| | 2) Evaluate the feedback from those Centres that completed 5 years and assess the current relevance of the scope of their activities for renewal | February 2025 |
| | 3) Increase visibility of current Centres: ask to submit maximum of 5 bullet points to be added to their website entry under the title “How can we help?” | For September 2024 |

| Subject | Issue | Status and Action |
|---|---|---|
| | 4) Explore mechanisms to improve collaboration by bringing together the Centres with the same main focus area (currently six): involvement of industry or other partners for fundings | On-going |
| | 5) Develop a questionnaire to gather feedback from the Collaborating Centres on their experiences being a WOAH CC, similar to the one for the Reference Laboratories | September 2024 |
| **Reference Laboratories** | 1) Put under-performing laboratories on watch list and monitor their performance. | On-going |
| | 2) Implement the new system for evaluating annual reports and provide list of assigned reports to Commission members | Accomplished |
| | 3) Send feedback to the Reference Laboratory network on the questionnaire | Accomplished |
| | 4) Explore enhancements to the annual report process: the possibility of filling in the annual report template throughout the year | May 2024 |
| **Reference Centre Networks** | 1) Follow up with the three Reference Laboratory networks (ASF, PPR and rabies) | On-going |
| **Standardisation/ Harmonisation** | 1) Project to extend the list of WOAH-approved reference reagents | |
| | a) Ask the other networks if they accept the minimum standards document proposed by PPR network. Once finalised, upload the document for implementation | For September 2024 |
| | 2) Project to develop Replacement International Standard Bovine and Avian Tuberculin: finalise report and propose for adoption | On-going |
| ***Ad hoc* Groups** | 1) *Ad hoc* Group on Sustainable Laboratories | On-going |
| | 2) Contribute on the review on the *Terrestrial Code* Chapter 4.7. Collection and processing of bovine, small ruminant and porcine semen | On-going |
| | 3) Contribute to the *Ad hoc* Group on Emerging Diseases and Drivers of Disease Emergence in Animals | On-going |
| **Projects** | 1) Veterinary Biobanking (project) | On-going |
| **Conferences, Workshops and Meetings with participation by BSC Members** | 1) Biosafety research roadmap | Accomplished |
| | 2) ISWAVLD WOAH Seminar, June 2025 in Canada: develop a theme and programme and speakers | September 2024 |
| **Performance** | 1) Engage with the ongoing processes around performance issues with Reference Laboratories | On-going |
| **Develop laboratory standards for emerging diseases** | 1) Discuss the *Terrestrial Code* chapter once adopted and consider introducing a corresponding chapter for the *Terrestrial Manual* | After May 2024 |
| **Case definitions** | 1) Follow up the implementation of the SOPs for case definitions | On-going |

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**Annex 4. Item 5.1. – Chapter 1.1.5. Quality management in veterinary testing laboratories**

**MEETING OF THE BIOLOGICAL STANDARDS COMMISSION**

**Paris, 5–9 February 2024**

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# 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~~ 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/IEC[1] 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. The work, responsibilities, and goals of the laboratory

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

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 WOAH;

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

## 3. Accreditation

If ~~the laboratory decides to proceed with~~ formal recognition of ~~its~~ a laboratory's quality management system and testing~~, then~~ is sought, third party verification of its conformity with the selected standard(s) ~~will be~~ is necessary. ILAC has published specific requirements and guides for laboratories and accreditation bodies. Under the ILAC system, ISO/IEC 17025 is to be used for laboratory accreditation of testing or calibration activities. Definitions regarding laboratory accreditation may be found in ISO/IEC International Standard 17000: Conformity Assessment – Vocabulary and General Principles (ISO/IEC, ~~2004a~~ 2020). Accreditation is ~~tied to~~ dependent on demonstrated

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

competence, which ~~is~~ encompasses significantly more than having and following documented procedures. Providing a competent and customer-oriented service also ~~means that the laboratory~~ requires:

i) Adequate facilities and environmental controls;

ii) ~~Has~~ Appropriately qualified and trained personnel with a depth of technical knowledge commensurate with appropriate level of authority;

iii) ~~Has appropriate~~ Equipment ~~with planned~~ that is appropriately verified and managed in accordance with the relevant maintenance and calibration schedule;

~~iv) Has adequate facilities and environmental control;~~

~~v) Has procedures and specifications that ensure accurate and reliable results;~~

~~vi) Implements continual improvements in testing and quality management;~~

~~vii) Can assess the need for and implement appropriate corrective or preventive actions, e.g. customer satisfaction;~~

~~viii) Accurately assesses and controls uncertainty in testing;~~

iv) Appropriate sample and materials management processes;

v) ~~Has~~ Technically valid and validated test methods, procedures and specifications~~ that are~~, documented in accordance with the requirements of the applicable standard or guidelines, e.g. Chapter 1.1.6 *Principles and methods of validation of diagnostic assays for infectious diseases*~~ and~~, chapters 2.2.1 to 2.2.8 *Recommendations for validation of diagnostic tests* and Special Issue of the *Scientific and Technical Review* (2021)[3];

vi) ~~Demonstrates~~ Demonstrable proficiency in the applicable test methods ~~used~~ (e.g. by regular participation in proficiency ~~tests on a regular basis~~ testing schemes);

vii) Accurate assessment and control of the measurement of uncertainty in testing;

viii) Good documentation practices, e.g. ALCOA+ principles (i.e. Attributable, Legible, Contemporaneous, Original, Accurate, Complete, Consistent, Enduring, Available);

ix) Non-conformance management process, including detection, reporting, risk-assessment and implementation of effective corrective and preventive actions;

x) Complaints management;

xi) Adequate control of data and information;

xii) Appropriate reporting and approval process;

xiii) Culture of continual improvement.

xiv) Has demonstrable competence to generate technically valid results.

## 4. Selection of an accreditation body

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

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

[3] Available at: https://doc.woah.org/dyn/portal/index.xhtml?page=alo&aloId=41245

## 5. Determination of the scope of the quality management system or of the laboratory's accreditation

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

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

~~i) The impact of initial accreditation on resources within a given deadline;~~

i) Associated risks and opportunities;

ii) Initial investment required (e.g. time, resources);

iii) ~~A~~ Contractual requirement for accredited testing (e.g. for international trade, research projects);

iv) ~~The~~ Importance of the test and the potential impact of an incorrect result;

v) The cost of maintaining an accredited test versus frequency of use;

vi) Availability of personnel, facilities and equipment;

vii) Availability of appropriate materials and reference standards (e.g. ~~standardised~~ reagents, ~~internal quality control samples~~ controls, reference cultures) ~~and~~

viii) Access to proficiency testing schemes;

ix) The quality ~~assurance~~ control processes necessary for materials, reagents and media;

x) The validation status, e.g. access to field samples from infected and non-infected animals, technical complexity and reliability of the test method;

xi) ~~The~~ Potential for subcontracting of accredited tests.

## 6. Quality assurance, quality control and proficiency testing

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

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

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

All laboratories should, where possible, participate in external proficiency testing schemes appropriate to ~~their testing. Participation~~ the suite of tests provided; participation in such schemes is a requirement for accredited

laboratories. This provides an independent assessment of the testing methods used ~~and~~ as well as the level of staff competence. If such schemes are not available, valid alternatives may be used, such as ring trials organised by reference laboratories, inter-laboratory testing, use of certified reference materials or internal quality control samples, replicate testing using the same or different methods, retesting of retained items, ~~and~~ or correlation of results for different characteristics of a specimen.

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

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

## 7. Test methods

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

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

In ~~the~~ veterinary ~~profession~~ laboratories, other standard methods (published in international, regional, or national standards) or fully validated methods (having undergone a full collaborative study and that are published or issued by an authoritative technical body such as the AOAC International) may be preferable to use, but ~~may~~ not ~~be~~ available. Many veterinary laboratories develop or modify methods, and most laboratories have test systems that use non-standard methods, or a combination of standard and non-standard methods. In veterinary laboratories, even with the use of standard methods, some in-house evaluation, optimisation, or validation is generally ~~must be done~~ required to ensure valid results.

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

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

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

### 7.1. Selection of the test method

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

#### 7.1.1. Considerations for the selection of a test method

i) International acceptance;

ii) Scientific acceptance;

iii) Appropriate or current technology;

iv) Suitable performance characteristics (e.g. analytical and diagnostic sensitivity and specificity, repeatability, reproducibility, isolation rate, limits of detection, precision, trueness, and uncertainty);

v) Suitability of the test in the species and population of interest;

vi) Sample type (e.g. serum, tissue, milk) and its expected quality or state on arrival at the laboratory;

vii) Test target (e.g. antibody, antigen, live pathogen, nucleic acid sequence);

viii) Test turnaround time;

ix) Resources and time available for development, adaptation, evaluation;

x) Intended use (e.g. export, import, surveillance, screening, diagnostic, confirmatory);

xi) Safety ~~factors~~ and biocontainment requirements;

xii) Customer expectations;

xiii) ~~Throughput of test~~ Sample numbers and required throughput (automation, robot);

xiv) Cost of test, per sample;

xv) Availability of reference standards, reference materials and proficiency testing schemes. (See also chapter 2.2.6.).

### 7.2. Optimisation and standardisation of the test method

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

#### 7.2.1. Determinants of optimisation

i) Critical specifications for equipment, ~~instruments~~ consumables, ~~and~~ reagents (e.g. chemicals, biologicals), reference standards, reference materials, and internal controls;

ii) Robustness – critical control points and acceptable ranges, attributes or behaviour at critical control points, using statistically acceptable procedures;

iii) Quality control activities necessary to monitor critical control points;

iv) The type, number, range, frequency, and arrangement of test run controls;

v) Criteria for ~~non-subjective~~ objective acceptance or rejection of ~~a batch of~~ test results;

vi) Criteria for ~~the~~ interpretation and reporting of test results;

vii) ~~A~~ Documented test method and reporting procedure ~~for use by laboratory staff~~;

viii) Evidence of technical competence for those ~~who~~ performing the test ~~processes~~ methods, authorising test results and interpreting results.

### 7.3. Validation of the test method

Test method validation evaluates the test for ~~its~~ fitness for ~~a given use~~ purpose by establishing ~~test~~ performance characteristics such as sensitivity, specificity, and isolation rate; and diagnostic parameters such as positive or negative cut-off, repeatability, reproducibility and titre of interest or significance. Validation should be ~~done~~ performed using an optimised, documented, and fixed procedure. The extent and depth of the validation process will depend on logistical and risk factors~~. It~~ and may involve any number of activities and amount of data, with subsequent data analysis using appropriate statistical methods (Chapter 1.1.6.). Acknowledging diagnostic test validation science as a key element in the effective detection of infectious diseases, WOAH recently published a Special Issue representing an up-to-date compilation of the relevant standards (WOAH and non-WOAH) and guidance documents for all stages of diagnostic test validation and proficiency testing, including design and analysis, as well as clear, complete and transparent reporting of validation studies in the peer-reviewed literature (Colling & Gardner, 2021). It is important to note that the current version of ISO 17025:2017 specifies that personnel must be authorised to perform validation and related activities, which means that training in validation and verification methods, including results interpretation, is likely to become more important to prove competence (Colling &

Gardner, 2021). It should also be noted that for veterinary laboratories, limited availability of suitable material may render validation difficult; under these circumstances it is necessary to highlight the limited validation status when reporting results and their interpretation (Stevenson *et al.*, 2021).

### 7.3.1. Activities that validation might include

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

~~ii) Development of testing algorithms for specific purposes, e.g. surveillance, outbreak investigations, etc.;~~

~~ii~~i) Repeat testing in the same laboratory to establish the effect of variables such as operator, reagents, equipment;

ii~~v~~) Comparison with other, preferably standard methods and with reference standards (if available);

iii~~v~~) Collaborative studies with other laboratories using the same documented method. Ideally organised by a reference laboratory and including testing a panel of samples of undisclosed composition or titre with expert evaluation of results and feedback to ~~the~~ participants to estimate reproducibility;

iv~~i~~) Reproduction of data from an accepted standard method, or from a ~~reputable~~ peer-reviewed publication (verification);

v~~ii~~) Experimental infection ~~or disease outbreak~~ studies;

vi~~ii~~) Analysis of internal quality control data.

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

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

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

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

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

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

## 7.4. Estimation of Measurement Uncertainty

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

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

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

The determination of MU is well established in *quantitative* measurement sciences (ANSI, 1997). It may be given as a numeric expression of reliability and is commonly shown as a stated range. Standard deviation (SD) and ~~confidence~~ reference interval (~~C~~RI) are examples of the expression of MU, for example the optical density result of an ELISA expressed as ± *n* SD, where *n* is usually 1, 2 or 3. The confidence interval (usually 95%) gives an estimated range in which the result is likely to fall, calculated from a given set of test data. For quantitative measurements, example for a top-down or control-sample approach are provided for an antibody ELISA in chapter 2.2.4, and by the Australian government webpage[4]. An example for a quantitative PCR hydrolysis probe ~~(TaqMan)~~ assay is provided by Newberry & Colling (2021).

The ISO/IEC 17025 requirement for "quality control procedures for monitoring the validity of tests" implies that the laboratory must use quality control procedures that cover all major sources of uncertainty. There is no requirement to cover each component separately. Laboratories may establish acceptable specifications, criteria, ranges, etc., at critical control points for each component of the test process. The laboratory can then implement appropriate quality control measures at these critical points, or seek to reduce or eliminate the uncertainty effect of each component.

### 7.4.1. Potential sources of uncertainty include:

i) Sampling;

ii) Contamination;

iii) Sample transport and storage conditions;

iv) Sample processing;

v) Reagent quality, preparation and storage;

vi) Type of reference material;

vii) Volumetric and weight manipulations;

viii) Environmental conditions;

ix) Equipment effects;

x) Analyst or operator bias;

xi) Biological variability;

xii) Unknown or random effects.

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

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

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

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

~~The ISO/IEC 17025 requirement for "quality control procedures for monitoring the validity of tests" implies that the laboratory must use quality control procedures that cover all major sources of uncertainty. There is no requirement to cover each component separately. Laboratories may~~

[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).

~~establish acceptable specifications, criteria, ranges, etc., at critical control points for each component of the test process. The laboratory can then implement appropriate quality control measures at these critical points, or seek to reduce or eliminate the uncertainty effect of each component. Measurement Uncertainty is covered in chapter 2.2.4.~~

#### ~~7.4.1. Components of tests with sources of uncertainty include:~~

~~i) Sampling;~~

~~ii) Contamination;~~

~~iii) Sample transport and storage conditions;~~

~~iv) Sample processing;~~

~~v) Reagent quality, preparation and storage;~~

~~vi) Type of reference material;~~

~~vii) Volumetric and weight manipulations;~~

~~viii) Environmental conditions;~~

~~ix) Equipment effects;~~

~~x) Analyst or operator bias;~~

~~xi) Biological variability;~~

~~xii) Unknown or random effects.~~

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

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

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

### 7.5. Implementation and use of the test method

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

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

## 8. Strategic planning

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

i) Attendance at conferences, organisation of in-house or external meetings on diagnostics and quality management;

ii) ~~Participation in~~ Membership of local and international organisations;

iii) ~~Participation in writing~~ Contribution to national and international standards (e.g. on ILAC and ISO committees);

iv) Maintenance of current awareness ~~of publications, writing~~ through review of and ~~reviewing publications about diagnostic methods~~ contribution to relevant literature;

v) Participation in training programmes, including visits to other laboratories;

vi) Conducting research;

vii) Participation in cooperative programmes (e.g. Inter-American Institute for Cooperation in Agriculture);

viii) Exchange of procedures, methods, reagents, samples, personnel, and ideas;

ix) Planned, continual professional development and technical training;

x) Management reviews;

xi) Analysis of customer feedback;

xii) Root cause analysis of anomalies and implementation of corrective, preventive and improvement actions, as well as effectiveness reviews.

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453 * *

454 NB: FIRST ADOPTED IN 1996 AS *GOOD LABORATORY PRACTICE, QUALITY CONTROL AND QUALITY ASSURANCE*.
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**Annex 5. Item 5.1. – Chapter 1.1.9. Tests for sterility and freedom from contamination of biological materials intended for veterinary use**

**MEETING OF THE BIOLOGICAL STANDARDS COMMISSION**

**Paris, 5–9 February 2024**

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

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

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

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

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

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

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

It is the responsibility of the submitter to ~~assure~~ ensure a representative selection and number of items to be tested. ~~The principles of~~ Appendix 1.1.2.1 Epidemiological approaches to sampling: sample size calculations of Chapter 1.1.2 Collection, submission and storage of diagnostic specimens ~~apply~~ describes the principles to be applied. ~~Adequate transportation is described in~~ Chapter 1.1.2 and Chapter 1.1.3 Transport of biological materials describe transportation requirements.

## A. AN OVERVIEW OF TESTING APPROACHES

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

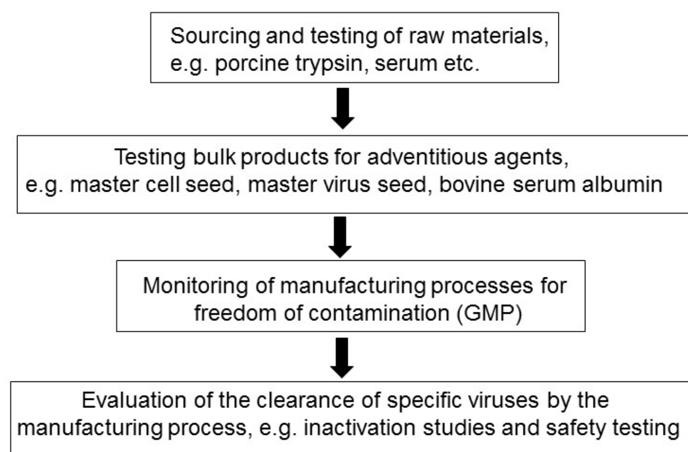
- Primary materials must be collected from sources shown to be free from contamination and handled in such a way as to minimise contamination and the opportunities for any contaminants to multiply (Figure 1).

- Materials that are not sterilised and those that are to be processed further after sterilisation must be handled aseptically. Such materials will require further assessment of freedom of contaminants at certain stages of production to assure freedom of adventitious agents.

- Materials that can be sterilised without their biological activities being affected unduly must be sterilised by a method effective for the pathogens ~~concerned~~ of concern. The method must reduce the level of contamination to be undetectable, as determined by an appropriate sterility test study. ~~(See Section D.1. below).~~ If a sterilisation process is used, it shall be validated to demonstrate that it is fit for purpose. Suitable controls will be included in each sterilisation process to monitor efficiency.

- The environment in which any aseptic handling is carried out must be maintained in a clean state, protected from external sources of contamination, and controlled to prevent internal contamination. Rules governing aseptic preparation of vaccines are documented in Chapter 2.3.3 *Minimum requirements for the organisation and management of a vaccine manufacturing facility*.

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




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

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

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

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

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

1. Materials of animal origin ~~shall~~ should be ~~(a)~~ sterilised~~, or (b)~~ and obtained from healthy animals that, in so far as is possible, should be shown to be free from pathogens that can be transmitted from the species of origin to the species to be vaccinated, or any species in contact with them by means of extraneous agents testing.

2. Seed lots of virus, any continuous cell line and biologicals used for virus growth ~~shall~~ should be shown to be free from ~~viable~~ bacteria, fungi, mycoplasmas, protozoa, rickettsia, and extraneous viruses ~~and other pathogens~~ that can be transmitted from the species of origin to the species to be vaccinated or any species in contact with them. ~~There may be some exceptions for a limited number of non-pathogenic bacteria and fungi to be present in live viral vaccines produced in eggs and administered through drinking water, spray, or skin scarification.~~

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

3. Each batch of vaccine ~~shall~~ should pass tests for freedom from extraneous agents that are consistent with the importing country's requirements for accepting the vaccine for use. Some examples of published methods that document acceptable testing ~~procedures~~ processes in various countries include: ~~(US) Code of Federal Regulations (2015); European Pharmacopoeia (2014); European Commission (2006); World Health Organization (WHO) (1998; 2012) and Department of Agriculture (of Australia) (2013).~~
   - Code of Federal Regulations, Title 9 (9CFR) (of the United States of America) (2015).
   - Department of Agriculture, Forest and Fisheries (Australia) (2013).
   - Department of Agriculture, Forest and Fisheries (Australia) (2021b) Live Veterinary Vaccines.
   - Regulation on Veterinary Drug Administration (China [People's Republic of]) (2020).
   - European Medicines Agency Sciences Medicines Health (2016).
   - European Pharmacopoeia, 10th Edition (2021).
   - World Health Organization (WHO) (1998; 2012).

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

## ~~C. LIVING VIRAL VACCINES FOR ADMINISTRATION THROUGH DRINKING WATER, SPRAY, OR SKIN SCARIFICATION~~

~~1. Section B applies.~~

~~2. A limited number of contaminating, non-pathogenic bacteria and fungi may be permitted (see Section I.2.2 *General Procedure for testing live viral vaccines produced in eggs and administered through drinking water, spray, or skin scarification for the presence of bacteria and fungi*).~~

## ~~D~~ C. INACTIVATED VIRAL AND BACTERIAL VACCINES

1. Each batch of vaccine shall pass a test for inactivation of the vaccinal ~~virus~~ seed and should include inactivation studies on representative extraneous agents if the virus or bacterial seed has not already been tested and shown to be free from extraneous agents. An example of a simple inactivation study could include assessment of the titre of live vaccine before and after inactivation and assessing the $\log_{10}$ drop in titre during the inactivation process. This would give an indication of the efficacy of the inactivation process. There is evidence that ~~virus~~ titration tests may not have sufficient sensitivity to ensure complete inactivation. In these circumstances, a specific innocuity test would need to be developed and validated to be fit for increased sensitivity. To increase sensitivity more than one passage would be required depending on the virus or bacteria of concern. An example of this approach can be found at: https://www.aphis.usda.gov/animal_health/vet_biologics/publications/memo_800_117.pdf (accessed 25 July 2023).

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

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

## ~~E.~~ D. LIVING BACTERIAL VACCINES

1. See Section B ~~applies~~.

2. Seed lots of bacteria shall be shown to be free from other bacteria as well as fungi and mycoplasmas, protozoa, rickettsia, and extraneous viruses. Agents required for exclusion will be dependent on the country accepting the vaccine for use. Use of antibiotics to 'inactivate' the living bacterial seed or vaccine prior to exclusion of viruses and fungi is recommended to ensure testing in culture is sensitive. ~~Interference testing is recommended to ensure that the antibiotics used do not affect the growth of the extraneous virus or fungi that is being excluded. Sonication may also be useful~~

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

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

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

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

## ~~F. INACTIVATED BACTERIAL VACCINES~~

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

## ~~G~~ E. SERA, PLASMA AND DIAGNOSTIC AGENTS FOR ADMINISTRATION TO ANIMALS

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

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

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

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

~~5. Section B or D may apply if a virus is used in the production of the diagnostic agent; Section E or F may 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 exhaustive list of agents of concern or by any means required for exclusion by every country based on risk, they are ~~just~~ examples of infectious agents that are not culturable using general culturing procedures and require ~~a more~~ use of specialised culturing processes and specific detection ~~process by means of the indirect fluorescent antibody test, PCR or ELISA, where applicable~~ processes. Notably, some subtypes of an agent type may be detectable by general methods, and some may require specialised testing for detection. For example, bovine adenovirus subgroup 1 (serotypes 1, 2, 3 and 9) can be readily isolated using general methods (Vero cells) however bovine adenovirus subgroup 2 (serotypes 4, 5, 6, 7, 8 and 10) are not readily isolated and required specialised methods for isolation.

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

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

## 2. Example of detection of bacteria and fungi ~~contamination~~

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

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

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

#### 2.1.1. Diluent A

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

#### 2.1.2. Diluent B

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

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

If direct inoculation of culture media is chosen, a sterile pipette or syringe and needle are used to aseptically transfer the biological material directly into liquid media. If the biological being tested has antimicrobial properties, the ratio of the inoculum to the volume of culture medium must be determined before the test is started, for example as explained in 9CFR 113.25(d) and detailed testing procedures can be found for example in supplemental assay method USDA SAM 903 https://www.aphis.usda.gov/animal_health/vet_biologics/publications/sam903.pdf (accessed 24 July 2023) ~~(SAM) 903 USDA SAM 903, See https://www.aphis.usda.gov/animal_health/vet_biologics/publications (accessed 4 July 2022)~~. To determine the correct medium volume to negate antimicrobial activity, 100 CFU of the control microorganisms listed in Table 2 are used. If the test sample contains merthiolate as a preservative, FTM is used in test vessels incubated at both 30–35°C and 20–25°C. Growth should be clearly visible after an appropriate incubation time (see Section I.2.1.3 *Growth promotion and test interference*). If the test sample is a killed biological without merthiolate, or a live bacterial biological, FTM is used at 30–35°C and SCDM at 20–25°C. If the test sample is a live viral biological, SCDM is used at both incubation temperatures. If the inactivated bacterial vaccine is a clostridial biological, or contains a clostridial component, the use of FTM with 0.5% added beef extract (FTMB) in place of FTM is preferred. It may also be desirable to use both FTM and SCDM for all tests.

***Table 2. Some American Type Culture Collection[1] strains with their respective medium and incubation conditions***

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

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

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

#### 2.1.3. Example of growth promotion and test interference

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

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

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

To test for ability of the culture media to support growth in the presence of the test material, containers should be inoculated simultaneously with both the test material and 10–100 viable control organisms. The number of containers used should be at least one-half the number used to test the product or product component. The test media are satisfactory if clear evidence of growth of the control organisms appears in all inoculated media containers within 7 days. In the event that growth is evident, the organism should be identified to confirm that it is the organism originally added to the medium. The sterility test is considered invalid if any of the media show inadequate growth response, or if the organism recovered, is not the organism used to inoculate the material.

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

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

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

### 2.~~3~~2. Example of general procedure for testing seed lots of bacteria and live bacterial biologicals for purity

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

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

If no atypical growth is found in any of the test vessels when compared with a positive control included in the test, the lot of biological may be considered satisfactory for purity. If atypical growth is found but

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

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

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

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

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

All plates and flasks are incubated at 37°C in a 5–10% $CO_2$ environment. Plates are incubated with the agar uppermost and flasks with the agar slope vertical. Flasks are incubated with the cap loose.

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

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

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

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

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

## 3. Example of detection of *Mycoplasma* ~~contamination~~

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

~~Each batch of live viral vaccine, each lot of master seed virus (MSV), each lot of primary and master cell stock (MCS), and all ingredients of animal origin not steam-sterilised should be tested for the absence of mycoplasmas. Solid and liquid media that will support the growth of small numbers of test organisms, such as typical contaminating organisms *Acholeplasma laidlawii*, *Mycoplasma arginini*, *M. fermentans*, *M. hyorhinis*, *M. orale*, and *M. synoviae* should be used. The nutritive properties of the solid medium should be such that no fewer than 100 CFU should occur with each test organism when approximately 100–200 CFUs are inoculated per plate. An appropriate colour change should occur in the liquid media when approximately 20–40 CFUs of each test organism are inoculated. The ability of the culture media to support growth in the presence of product should be validated for each product to be tested, and for each new batch or lot of culture media.~~

~~One sample of each lot of vaccine, e.g. MSV or MCS, should be tested. Four plates of solid medium are inoculated with 0.25 ml of the sample being tested, and 10 ml of the sample inoculated into 100 ml of the liquid medium. An alternative is to inoculate each of the plates with 0.1 ml and to inoculate 100 ml of liquid medium with 1 ml of the sample being tested. Two plates are incubated at 35–37°C aerobically (an atmosphere of air containing 5–10% $CO_2$ and adequate humidity) and two plates are incubated anaerobically (an atmosphere of nitrogen containing 5–10% $CO_2$ and adequate humidity) for 14 days. On day 3 or day 4 after inoculation, 0.25 ml from the liquid media are subcultured on to two plates of solid media. One plate is incubated aerobically and the second anaerobically at 35–37°C for 14 days. The subculture procedure is repeated on day 6, 7, or 8 and again on day 13 or 14. An alternative method is to subculture on days 3, 5, 10, and 14 on to a plate of solid medium. All the subculture plates are incubated for 10 days except for the 14-day subculture, which is incubated for 14 days. Liquid media is observed every 2–3 days and, if any colour change occurs, has to be subcultured immediately.~~

### ~~3.2. Interpretation of *Mycoplasma* test results~~

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

~~Further detailed procedures can be found in Veterinary Medicinal Products, VICH GL34: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2013/03/WC500140352.pdf~~

Prior to beginning testing it is necessary to determine that each batch of media promotes the growth of *M. mycoides* subsp. *mycoides* ~~SC~~ (*Mmm*~~SC~~) type strain PG1. General mycoplasma broth and agar are used but contain porcine serum as a supplement. Each batch of broth and agar is inoculated with 10–100 CFU of *Mmm*~~SC~~. The solid medium is suitable if adequate growth of *Mmm*~~SC~~ is found after 3–7 days' incubation at 37°C in 5–10% $CO_2$. The liquid medium is suitable if the growth on the agar plates subcultured from the broth is found by at least the first subculture. If reduced growth occurs another batch of media should be obtained and retested.

1 ml of cell or virus seed to be tested is inoculated into 9 ml of the liquid medium and 100 µl on to solid mycoplasma agar. The volume of the product is inoculated so that it is not more than 10% of the volume of the medium. The liquid medium is incubated at 37°C in 5–10% $CO_2$ and 100 µl of broth is subcultured on to agar at days 7, 14 and 21. The agar plates are incubated at 37°C in 5–10% $CO_2$ for no fewer than 14 days, except those corresponding to day 21 subculture, which are incubated for 7 days. An un-inoculated mycoplasma broth and agar plate are incubated as negative controls. For assessment of inhibitory substances, inoculate 1 ml of sample to be tested into 9 ml of the liquid medium and 100 µl on to solid medium and add 10–100 CFU of *Mmm*~~SC~~ to each. Prepare positive control by inoculating 9 ml of mycoplasma broth and a mycoplasma agar plate with 10–100 CFU of *Mmm*~~SC~~. Incubate as for samples and negative controls.

During incubation time, visually compare the broth of the positive control with sample present with the positive control broth and, if there is no inhibition of the organism either the product possesses no antimicrobial activity under the conditions of the test, or such activity has been satisfactorily eliminated by dilution. If no growth or reduced growth of *Mmm*~~SC~~ is seen in the liquid and solid medium with test sample when compared with the positive control, the product possesses antimicrobial activity, and the test is not satisfactory. Modifications of the conditions to eliminate the antimicrobial activity and repeat test are required.

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

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

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

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

Further detailed procedures can be found in Veterinary Medicinal Products, VICH GL34: ~~http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2013/03/WC500140352.pdf~~ https://www.ema.europa.eu/en/vich-gl34-biologicals-testing-detection-mycoplasma-contamination-scientific-guideline

and

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

## 4. Example of detection of rickettsia and protozoa

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

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

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

## 5. Example of detection of virus ~~viruses in biological materials~~

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

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

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

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

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

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

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

Cell seed stocks do not require a neutralisation process.

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

#### 5.1.1 Example of amplification in cell culture

~~The cells should be passaged weekly up to a 28-day period.~~ Continuous and primary, 75 cm$^2$ area monolayers of the source species (and intended species as applicable) are infected with 1 ml of seed stocks and passaged weekly for ~~between~~ up to 21–28-days. Depending on the procedure followed, monolayers can be subcultured between passes or freeze/thawed to disrupt cells. Negative and positive controls should be also set up at each pass using the same cell population. Certain relevant viruses may be selected as indicators for sensitivity and interference (positive controls) but these will not provide validation for the broader range of agents targeted in general testing. The final culture is examined for cytopathology and haemadsorption.

#### 5.1.2 Example of general detection procedures: cytopathology

May–Grünwald–Giemsa or H&E staining procedures are used to assess for cytopathological changes associated with virus growth. Monolayers must have a surface area of at least 6 cm$^2$ and can be prepared on appropriate chambered tissue culture slides and incubated for 7 days. The plastic wells of the slides are removed leaving the rubber gasket attached to the slide. The slides are rinsed in Dulbecco's phosphate buffered saline (PBS), fixed in acetone, methanol or formalin depending on the stain used and placed on a staining rack. For May–Grünwald–Giemsa staining: the slides are stained for 15 minutes at room temperature with May–Grünwald stain diluted 1/5 with absolute methanol. The May–Grünwald stain is removed by inverting the slides. The slides are then stained for 20 minutes with Giemsa stain diluted 1/15 in deionised water. The Giemsa stain is removed by inverting the slides and rinsing them in deionised water for 10–20 seconds. The slides are air-dried and mounted with a coverslip using paraffin oil. The May–Grünwald–Giemsa stain differentially stains ribonucleoprotein (RNP); DNA RNP stains red-purple, while RNA RNP stains blue. The monolayers are examined with a conventional microscope for

the presence of inclusion bodies, an abnormal number of giant cells, or other cytopathology attributable to a viral contaminant of the test product. The inoculated monolayers are compared with suitable control non-inoculated monolayers. If specific cytopathology attributable to an extraneous virus is found, results are reported, and additional specific testing may be conducted.

#### 5.1.3 Example of general detection procedures: haemadsorption

Testing for haemadsorption ~~uses~~ requires the use of 75 $cm^2$ area monolayers established in tissue culture flasks after the 28-day passage period described above. Guinea-pig, chicken, and any other blood for use in this assay is collected in an equal volume of Alsever's solution and may be stored at 4°C for up to 7 days. Immediately prior to use, the stored erythrocytes are again washed by adding 5 ml of blood in Alsever's solution to 45 ml of calcium and magnesium-free PBS (PBSA) and centrifuging in a 50 ml centrifuge tube at 500 ***g*** for 10 minutes. The supernatant is aspirated, and the erythrocytes are suspended in PBSA and re-centrifuged. This washing procedure is repeated at least twice until the supernatant is clear. Erythrocytes from each species are combined by adding 0.1 ml of each type of packed blood cells to 100 ml of PBSA. The erythrocytes from different species may be kept separate or combined, as desired. To each flask, add 5 ml of the erythrocyte suspension, and incubate the flasks at 4°C for 30 minutes. Monolayers are washed twice with PBSA and examined for haemadsorption. If no haemadsorption is apparent, 5 ml of ~~the~~ fresh erythrocyte suspension is added to each flask; the flasks are incubated at 20–25°C (room temperature) for 30 minutes, rinsed as before, and examined for haemadsorption. Separate flasks may be used for each incubation temperature if desired. Monolayers are examined for the presence of haemadsorption using an illuminated light box and microscopically. Non-inoculated monolayers are used as negative controls. The PBSA and fresh erythrocytes should prevent most nonspecific haemadsorption from occurring. If specific haemadsorption attributable to an extraneous agent is found, results are reported, and additional specific testing may be conducted.

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

### 5.2. ~~An~~ Examples of specific ~~virus~~ agent exclusion testing ~~from~~ of biologicals used in the production of veterinary vaccines

#### 5.2.1. Example of porcine epidemic diarrhoea virus (PEDV)

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

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

Add 100 µl of diluted virus on to each of two duplicate wells. Rock inoculated plates to distribute the inoculum evenly over the surface of the monolayer. Incubate at 37°C with 5% $CO_2$ for 2 hours then add a further 1 ml volumes/well of MM.

661 After 7 days, 75 $cm^2$ monolayers have cells disrupted using two freeze–thaw cycles at –80°C. Positive control plates are read for end-point titres, and these are compared with virus in the presence of test material to ensure titres are comparable and interference has not occurred. Freeze–thaw lysates are clarified at 2000 ***g*** for 5 minutes and re-passed on to newly formed monolayers as for the first passage. Passages are repeated until a total of four passages are completed at which point cell lysates are assessed by PCR for detection of PEDV and day 7 monolayers in 24-well plates are fixed and stained by IFA for FAT. If a seed virus is to be tested and requires neutralisation using antiserum, extra care in the isolation of PEDV needs to be considered. Trypsin is rendered inactive in the presence of serum proteins and without trypsin present, PEDV is unable to grow in cell culture grows poorly, or not at all. Washing off the inoculum with two MM washes is required after an extended adsorption time of up to 4 hours to ensure acceptable sensitivity.

## J H. INFORMATION TO BE SUBMITTED WHEN APPLYING FOR AN IMPORT LICENCE

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

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

- European Commission (2015). The Rules Governing Medicinal Products in the European Union. Eudralex. Volume 6. Notice to applicants and regulatory guidelines for medicinal products for veterinary use.
- Department of Agriculture, Forest and Fisheries of Australia (2021b). Live veterinary vaccines Summary of information required for biosecurity risk assessment, Version 6 and Inactivated veterinary vaccines, Version 8.
- Outline of the Regulatory System of Veterinary Drugs in Japan (2015) Assurance of the Quality, Efficacy, and Safety Based on the Law for Ensuring the Quality, Efficacy, and Safety of Drugs and Medical Devices.
- Ministry of Agriculture and Rural Affairs, China (People's Rep. of), Regulations on the Administration of Veterinary drugs (revised in 2020).

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

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

## I. RISK ANALYSIS PROCESS

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

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

707 ~~I.~~ J. BIOCONTAINMENT

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

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

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

# 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 ~~a~~n approximate~~ly equal to a~~ 95% ~~confidence~~ reference interval (~~C~~ RI), relative standard deviation (RSD = SD / mean of replicates) and coefficient of variation (CV = RSD × 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).

## 1. Samples for use in determining MU

Repeatability is the level of agreement between results of replicates of a sample both within and between runs of the same test method in a given laboratory. During assay development, repeatability is estimated by evaluating variation in results of independent replicates from a minimum of three (preferably five) samples representing analyte activity within the operating range of the assay (see ~~the WOAH Validation Standard,~~ Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*, Sections A.2.5 *Robustness* and B.1.1 *Repeatability*, and Chapter 2.2.6 *Selection and use of reference samples and panels*, Section ~~3.1~~ A.4.2). Typically, the variation in replicate results is expressed as RSD or CV. The significant feature is that repeatability studies can be used to define the expected precision of the assay in the detection of a range of analyte concentrations.

The use of internal quality or process controls over a range of expected results has become part of daily quality control and quality assurance operations of accredited facilities (see ~~the WOAH Validation Standard,~~ chapter 1.1.6, Sections A.2.6 *Calibration of the assay to standard reagents* and B.5.1 *Monitoring the assay*, and Chapter 2.2.6, Section ~~1.4~~ C.1). These results provide a continuous monitor relative to different aspects of repeatability, e.g. intra- and inter-assay variation, intra- and inter-operator variation and intra- and inter-batch variation, which, when subjected to statistical analysis, provide an expression of the level of robustness (precision) of a test procedure. The monitoring of assay quality control parameters for repeatability provides evidence that the assay is or is not performing as expected. For control samples to provide valid inferences about assay precision, they should be treated in exactly the same way as test samples in each run of the assay, e.g. including sample preparation such as extraction steps or dilution of serum samples for an antibody enzyme-linked immunosorbent assay (ELISA).

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

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

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

## 2. Example of MU calculations in ELISA serology

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

A limited data set from a competitive ELISA for antibody to avian influenza virus is used as an example of a "top-down" approach for serology. A ~~low~~ weak positive control sample was used to calculate MU at the cut-off level[1].

### 2.1. Method of expression of MU

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

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

[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)

79 X represents the set of replicates

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

89 $$PI_{LW} = 100 \times [1 - \{OD_{LW} / OD_N\}]$$

90 The relative standard deviation becomes:

91 $$RSD\ (PI_{LW}) = SD\ (PI_{LW}) / mean\ (PI_{LW})$$

92 ### 2.2. Example

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

97 ***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 |

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

99 ### 2.3. Calculating uncertainty

100 From the limited data set,

101 $RSD\ (PI_{LW}) = SD/Mean = 7.9/56.3 = 0.14$ (or as coefficient of variation = 14%)

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

107 U (95% C RI) = 2 × RSD = 0.28

108 This estimate can then be applied at the threshold level

95% ~~C~~RI = 50 ± (50 × 0.28) = 50 ± 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*

| *Test* | *Ct value* |
|---|---|
| 1 | 33.60 |
| 2 | 33.20 |
| 3 | 33.96 |
| 4 | 33.18 |
| 5 | 33.96 |
| 6 | 32.72 |
| 7 | 33.57 |
| 8 | 33.45 |
| 9 | 32.80 |
| 10 | 33.20 |

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

### 3.2. Calculating uncertainty

From the limited data set,

RSD (PI~~L~~W) = SD/Mean 0.43/33.36 = 0.0128 (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~~L~~W) 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 (95% ~~C~~RI) = 2 × RSD = 0.0255

This estimate can then be applied at the threshold level

95% ~~C~~RI = 37± (37 × 0.0255) = 37 ± 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~~RI) 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. geomean 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.

### Scope and limitations of the top-down approach

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

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

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

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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 | Group B | Group D |
|---|---|---|
| **Proof of concept,** A.2.1. | **Asp,** B.1.2. | **Standard method comparison,** B.2.6. |
| **Operating range,** A.2.~~2~~ 3. | **Analytical accuracy, ancillary tests** B.1.4. | **Provisional recognition, B.**2.~~6~~ 7. |
| **ASe,** B.1.3. | ~~**Reference samples and panels**~~ | **Biological modifications,** B.5.2.2. |
| **Optimisation,** A.2.~~3~~ 2. | **Group C** | **Group E** |
| ~~**Robustness,** A.2.5.~~ **Preliminary repeatability,** A.2.8. | **Repeatability** B.1.1. | **DSp and DSe Gold standard,** B.2.1. |
| **Calibration and process control,** A.2.6. | **Preliminary reproducibility, B.**2.~~6~~ 7. | **Group F** |
| ~~**Process control,** A.2.6.~~ | **Reproducibility,** B.3. | **DSp and DSe no gold standard** B.2.2. |
| ~~**ASe,** B.1.3.~~ | **Proficiency testing,** B.5.1. | |
| **Technical modifications,** B.5.2.1. | | |
| **Reagent replacement,** B.5.2.3. | | |

*ASp = Analytical specificity; ASe = Analytical sensitivity; DSp = diagnostic specificity; DSe = diagnostic sensitivity*

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

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

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

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

57 ***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.*

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

| Pathogen data | Animal host and sample type data | Phase of Infection data |
|---|---|---|
| • Strain/isolate<br>• Serotype<br>• Genotype<br>• Lineage<br>• Tests used for characterisation | • Natural infection<br>• Experimental infection and protocol used<br>• Species<br>• Breed<br>• Age<br>• Sex<br>• Reproductive status<br>• Vaccination history<br>• Herd history | • Clinical signs<br>• infection/disease outcome<br>• Antibody profiles<br>• Pathogen loading and shedding<br>• Tests used to determine status of disease/infection (case definition)<br>• Time post-experimental infection |
| | • Tissue type/s (matrix) used<br>• For spiked samples – detail source of analyte and diluent (matrix) used<br>• Details relating to pooling of samples | |

# A. GROUP A

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

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

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

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

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

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

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

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

## 2. Operating range (~~WOAH Validation Standard,~~ Chapter 1.1.6, Section A.2.~~2~~ 3) and analytical sensitivity (~~WOAH Validation Standard,~~ Chapter 1.1.6, Section B.1.3)

### 2.1. ~~Analytical approaches~~ Operating range and analytical sensitivity

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

### 2.2. Comparative approaches to analytical sensitivity

If the intended purpose is to detect low levels of analyte or subclinical infections, it may be difficult to obtain the appropriate reference materials from early stages of the infection process. In some cases, it may be useful to determine a comparative ASe by running a panel of samples on the candidate assay and on another independent assay. Ideally this panel of samples would be serially collected from either naturally or experimentally infected animals and should represent infected animals early after infection, ~~on~~ through to the development of clinical or fulminating disease, if possible. This would provide a relative comparison of ASe between the assays~~, as well as,~~ and a temporal comparison of the earliest point of detection relative to the pathogenesis of the disease.

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

## 3. Optimisation (~~WOAH Validation Standard,~~ Chapter 1.1.6, Section A.2.~~3~~2) and preliminary repeatability (~~WOAH Validation Standard,~~ Chapter 1.1.6, Section A.2.~~6~~8)

Optimisation is the process by which the most important physical, chemical and biological parameters of an assay are evaluated and adjusted to ensure that the performance characteristics of the assay are best suited to the intended application. At least three reference samples representing negative, ~~low~~ weak and ~~high~~ strong positive may be chosen from either natural or prepared reference samples. Optimisation experiments are rather exhaustive especially when assays

with multiple preparatory and testing steps are involved. It is very important that a sufficient quantity of each reference sample be available to complete all optimisation experiments. Changing reference samples during the course of optimisation is not recommended as this will result in the addition of an uncontrolled variable and a disruption in the continuity of optimisation evidence.

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

## 4. Calibration and process controls (~~WOAH Validation Standard,~~ Chapter 1.1.6, Section A.2.6)

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

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

Reference standards, especially antibody, are usually provided in one of two formats. They may be provided as a single positive reagent of given titre with the expectation that the candidate assay will be standardised to give an equivalent titre. This is a straight forward analytical approach but many of these ‘single’ standards have been prepared from highly positive samples as a pre-dilution in a negative matrix in order to maximise the number of aliquots available. The drawback here is that there is no accounting for any potential matrix effect in the candidate assay as there is no matrix control provided. The other approach is to provide a negative and a ~~low~~ weak and ~~high~~ strong positive set of reference standards that are of known concentrations or reactivities and are within the operating range of the standard method that was used to prepare them. The negative provided in the set must be the same as the negative diluent used to prepare the weak and strong positive reference standard, if the positive standards were diluted. This compensates for any potentially hidden matrix effect. In addition, this set of three acts as a template for the selection and/or preparation of process controls (discussed below).

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

### 4.2. Working standards or process controls

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

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

Technical modifications to a validated assay such as changes in instrumentation, extraction protocols, and conversion of an assay to a semi-automated or fully automated system using robotics will typically not necessitate full revalidation of the assay. Rather, a methods comparison study may be done to determine if these minor modifications to the assay protocol

205 will affect the test results. Consult See chapter 2.2.8 *Comparability of assays after changes in a validated test method* for
206 description of experiments and statistical approaches to assay precision in the face of technical modifications that are
207 appropriate for comparability testing (Bowden & Wang, 2021; Reising *et al.,* 2021).

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

## 216 6. Reagent replacement (WOAH Validation Standard, Chapter 1.1.6, Section B.5.2.3)

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

221 Again, it cannot be over-emphasised that any Replacement reference reagent should be selected or prepared using the
222 same criteria or preparation procedures established for previous materials. Again as this enhances the continuity of
223 evidence and confidence in the assay and underlines the importance of documentation of reference material data (Figure
224 2).

# 225 B. GROUP B

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

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

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

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

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

251 A second element, sometimes termed 'exclusivity', Exclusivity is the capacity of the assay to detect an analyte or genomic
252 sequence that is unique to a targeted organism, and excludes all other other known organisms that are potentially cross-
253 reactive. This is especially true in serological assays where there are many examples of antigens expressed by other
254 organisms that are capable of eliciting cross-reacting antibody. An attempt should be made to obtain reference samples
255 from documented cases of infections and/or organisms that may be cross-reactive. Depending on the type of assay, these

reference materials may represent the organism itself, host-derived samples, or genomic sequences. A profile for the exclusivity of the assay should be established, and expanded on a continual basis as potentially cross-reactive organisms arise.

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

## 2. Analytical accuracy of ~~adjunct~~ ancillary tests (~~WOAH Validation Standard,~~ Chapter 1.1.6, Section B.1.4)

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

# C. GROUP C

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

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

## 1. Repeatability (~~WOAH Validation Standard,~~ Chapter 1.1.6, Section B.1.1) and ~~preliminary reproducibility~~ provisional assay recognition (~~WOAH Validation Standard,~~ Chapter 1.1.6, Section B.2.6)

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

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

## 2. Reproducibility (~~WOAH Validation Standard,~~ Chapter 1.1.6, Section B.3)

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

## 3. Proficiency testing (~~WOAH Validation Standard,~~ Chapter 1.1.6, Section B.5.1)

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

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

# D. GROUP D

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

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

## 1. Standard method comparison and provisional recognition (~~WOAH Validation Standard,~~ Chapter 1.1.6, Sections B.2.~~6~~ 5 and B.2.6)

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

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

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

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

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

## E. GROUP E

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

### 1. 'Gold standard'[2] – diagnostic specificity and diagnostic sensitivity (~~WOAH Validation Standard,~~ Chapter 1.1.6, Section B.2.1)

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

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

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

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

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

## F. GROUP F

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

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

Reference populations, not individual reference samples, used in latent-class studies need to be well described~~. This includes documentation on both the pathogen and donor host. For pathogens, this may include details related to strain, serotype, genotype, lineage, etc., that may be circulating in the population. The source of the host material should be well described with respect to species, breed, age, sex, reproductive status, vaccination history, herd history, etc.~~ as summarised in Figure 2. Wherever possible, the phase of infection in the populations should be noted with respect to morbidity or mortality events, recovery, etc.

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

## FURTHER READING


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464 *
465 * *

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

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

1 C H A P T E R 3 . 1 . 5 .

# 2 CRIMEAN–CONGO HAEMORRHAGIC FEVER

## 3 SUMMARY

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

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

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

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

38 (*PCR*) *can be used. As infections of animals remain clinically unapparent, the likelihood of isolating virus*
39 *from a viraemic animal is very low.*

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

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

46 
## A. INTRODUCTION

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

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

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

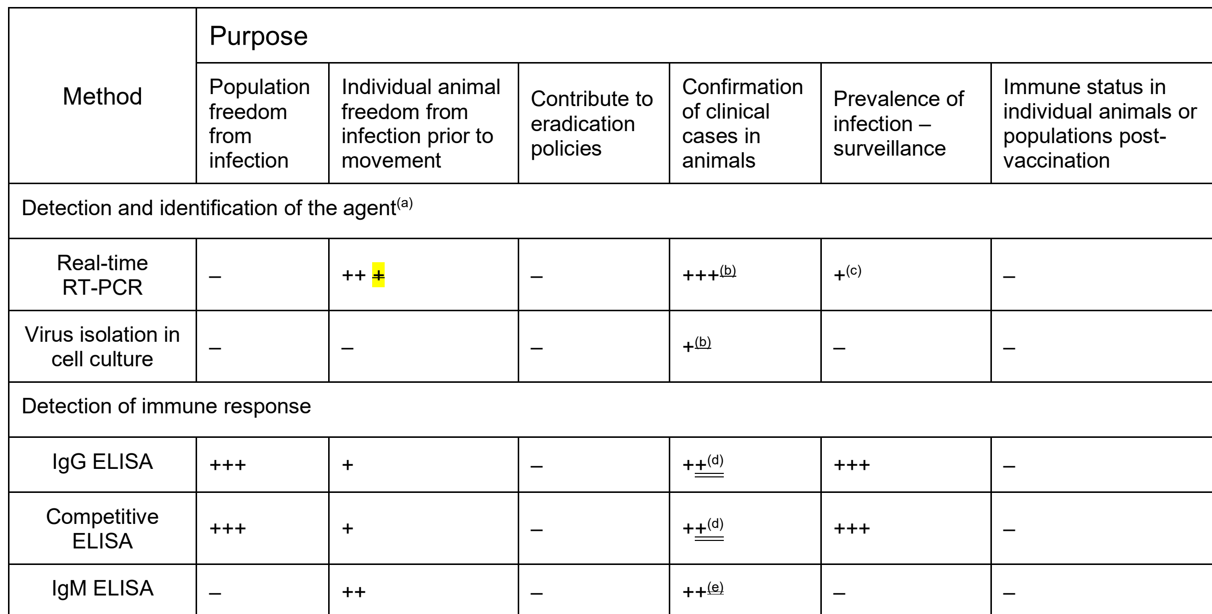
72 Humans acquire infection from tick bites, or from contact with infected blood or tissues from livestock or human patients.
73 After incubation humans can develop a severe disease with a prehaemorrhagic phase, a haemorrhagic phase, and a
74 convalescence period. Haemorrhagic manifestations can range from petechiae to large haematomas. Bleeding can be
75 observed in the nose, gastrointestinal system, uterus and urinary tract, and the respiratory tract, with a case fatality rate
76 ranging from 5% to 80% (Ergonul, 2006; Yen ***et al.,*** 1985; Yilmaz ***et al***., 2008).The severity of CCHF in humans highlights
77 the impact of this zoonotic disease on public health. Although CCHFV has no economic impact on livestock animal
78 production, the serological screening of animal serum samples for CCHFV-specific antibodies is very important. As
79 seroprevalence in animals is a good indicator for local virus circulation, such investigations allow identification of high-risk
80 areas for human infection (Mertens ***et al.,*** 2013). Slaughterhouse workers, veterinarians, stockmen and others involved
81 with the livestock industry should be made aware of the disease. They should take practical steps to limit or avoid exposure
82 of naked skin to fresh blood and other animal tissues, and to avoid tick bites and handling ticks. Experiences from South
83 Africa demonstrated that the use of repellents on animals before slaughter could reduce the numbers of infected
84 slaughterhouse workers (Swanepoel ***et al.,*** 1998). The treatment of livestock in general can reduce the tick density among
85 these animals and thus reduce the risk of tick bite in animal handlers (Mertens ***et al.,*** 2013). Such tick control by the use
86 of acaricides is possible to some extent, but may be difficult to implement under extensive farming conditions. Inactivated
87 mouse brain vaccine for the prevention of human infection has been used on a limited scale in Eastern Europe and the
88 former USSR (Swanepoel & Paweska, 2011). Progress in CCHFV vaccine development is being made with several
89 different approaches trialled to overcome current challenges (Dowall ***et al.,*** 2017).

90 Infectivity of CCHFV is destroyed by boiling or autoclaving and low concentrations of formalin or beta-propriolactone. The
91 virus is sensitive to lipid solvents. It is labile in infected tissues after death, presumably due to a fall in pH, but infectivity is
92 retained for a few days at ambient temperature in serum, and for up to 3 weeks at 4°C. Infectivity is stable at temperatures

below –60°C (Swanepoel & Paweska, 2011). CCHFV should be handled with appropriate biocontainment measures determined by risk analysis as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities* (Palmer, 2011; Whitehouse, 2004).

# B. DIAGNOSTIC TECHNIQUES

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



| Method | Purpose | | | | | |
|---|---|---|---|---|---|---|
| | Population freedom from infection | Individual animal freedom from infection prior to movement | Contribute to eradication policies | Confirmation 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)] | – | – |

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
RT-PCR = reverse-transcription polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended.
[(b)]Molecular testing/isolation can be used to confirm acute infection in rare cases in animals showing clinical signs as viraemia tends to be transient.
[(c)]RT-PCR is used for the screening of tick populations in the context of surveillance studies.
[(d)]Serological evidence of active infection with CCHFV has been demonstrated by seroconversion based on a rise in total or IgG antibody titres on samples taken at 2–4 weeks apart.
[(e)]Serological evidence of active infection with CCHFV has been demonstrated by the detection of IgM antibodies specific to

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

## 1. Detection and identification of the agent

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

The virus can be isolated from serum and organ suspensions in a wide variety of cell cultures, including Vero, LLC-MK2, SW-13, BSR-T7/5, CER and BHK21 cells, and identified by immunofluorescence using specific antibodies. Isolation and

identification of virus can be achieved in 1–5 days, but cell cultures lack sensitivity and usually only detect high concentrations of virus present in the blood.

## 1.1. Virus isolation in cell culture

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

### 1.1.1. Test procedure

i) Susceptible cell lines include Vero-E6, BHK-21, LLC-MK2 and SW-13 cells. Inoculate 80% confluent monolayers of the preferred cell line with the specimen. The volume of specimen to be used depends on the size of the culture vessel (i.e. 25 $cm^2$ culture flask or 6- or 24-well tissue culture plate). The specimen volume should be sufficient to cover the cell monolayer. Samples of insufficient volumes can be diluted with tissue culture medium to prepare sufficient inoculation volume.

ii) Adsorb the specimen for 1 hour at 37°C.

iii) Remove inoculum. Add fresh tissue culture medium containing 2% fetal calf serum and other required additives, as per specific medium and cell line requirements.

iv) Incubate at 37°C and 5% $CO_2$ for 4–7 days.

v) Test supernatant for presence of CCHFV viral RNA using real-time RT-PCR as described below, or perform immunofluorescence assay on cell scrapings.

vi) Isolates of CCHFV from clinical specimens cause no microscopically recognisable cytopathic effects (CPE) in most of these cell lines.

## 1.2. Nucleic acid detection

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

***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 CCRealP1 (TCT-TYG-CHG-ATG-AYT-CHT-TYC)<br>Rev CCRealP2 (GGG-ATK-GTY-CCR-AAG-CA)<br>Probe (ACA-SRA-TCT-AYA-TGC-AYC-CTG-C) |
| Africa 2 | Real-time RT-PCR | Fwd CCRealP1 (TCT-TYG-CHG-ATG-AYT-CHT-TYC)<br>Rev CCRealP2 (GGG-ATK-GTY-CCR-AAG-CA)<br>Probe (ACA-SRA-TCT-AYA-TGC-AYC-CTG-C) |
| | Real-time RT-PCR | Fwd CCHF-SF2 (GGA-VTG-GTG-VAG-GGA-RTT-TG)<br>Rev CCHF-SR2 (CAD-GGT-GGR-TTG-AAR-GC)<br>Fwd CCHF-N2 (CAA-RGG-CAA-RTA-CAT-MAT) |

| Clade | Molecular assay combinations | Primer and probe names (5’ → 3’ sequence) |
|---|---|---|
| Africa 3 | Nested RT-PCR | Fwd CCHF1 (CTG-CTC-TGG-TGG-AGG-CAA-CAA)<br>Rev CCHF2_5 (TGG-GTT-GAA-GGC-CAT-GAT-GTA-T)<br>Nested Fwd CCHFn15 (AGG-TTT-CCG-TGT-CAA-TGC-AAA)<br>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)<br>Rev CrCon1– (TRG-CAA-GRC-CKG-TWG-CRA-CWA-GWG-C)<br>Nested Fwd CriCon2+ (ART-GGA-GRA-ARG-AYA-TWG-GYT-TYC-G)<br>Nested Rev CriCon2– (CYT-TGA-YRA-AYT-CYC-TRC-ACC-ABT-C) |
| Africa 4 | Real-time RT-PCR | Fwd CCRealP1 (TCT-TYG-CHG-ATG-AYT-CHT-TYC)<br>Rev CCRealP2 (GGG-ATK-GTY-CCR-AAG-CA)<br>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)<br>Rev CCHF-III-r (GCC-ACG-GGG-ATT-GTC-CCA-AAG-CAG-AC)<br>Probe CCHFprobe-1 (ATC-TAC-ATG-CAC-CCT-GCY-GTG-YTG-ACA)<br>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)<br>Fwd CCF-131F (TGG-AYA-CYT-TCA-CAA-ACT-CC)<br>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)<br>Rev CC1a_rev (GTG-CCA-CTG-ATG-ATG-CAC-AAA-AGG-ATT-CCA-TCT)<br>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)<br>Rev CCHF-SR2 (CAD-GGT-GGR-TTG-AAR-GC)<br>Probe CCHF-N2 (CAA-RGG-CAA-RTA-CAT-MAT) |
| Asia 2 | Nested PCR | Fwd CCF-115F (AAR-GGA-AAT-GGA-CTT-RTG-GA)<br>Fwd CCF-131F (TGG-AYA-CYT-TCA-CAA-ACT-CC)<br>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)<br>Rev (GTA-GAT-GGA-ATC-CTT-TTG-TGC-ATC-AT) |
| | RT-PCR | Fwd CCS (ATG-CAG-GAA-CCA-TTA-ART-CTT-GGG-A)<br>Rev 1 CCAS1 (CTA-ATC-ATA-TCT-GAC-AAC-ATT-TC)<br>Rev 2 CCAS2 (CTA-ATC-ATG-TCT-GAC-AGC-ATC-TC) |
| Europe 1 | Real-time RT-PCR | Fwd CCRealP1 (TCT-TYG-CHG-ATG-AYT-CHT-TYC)<br>Rev CCRealP2 (GGG-ATK-GTY-CCR-AAG-CA)<br>Probe (ACA-SRA-TCT-AYA-TGC-AYC-CTG-C) |
| | Nested RT-PCR | Fwd CCF-115F (AAR-GGA-AAT-GGA-CTT-RTG-GA)<br>Fwd CCF-131F (TGG-AYA-CYT-TCA-CAA-ACT-CC)<br>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)<br>Rev CrCon1– (TRG-CAA-GRC-CKG-TWG-CRA-CWA-GWG-C)<br>Fwd CriCon2+ (ART-GGA-GRA-ARG-AYA-TWG-GYT-TYC-G)<br>Rev CriCon2– (CYT-TGA-YRA-AYT-CYC-TRC-ACC-ABT-C) |
| Europe 3 | Real-time RT-PCR | Fwd CCRealP1 (TCT-TYG-CHG-ATG-AYT-CHT-TYC)<br>Rev CCRealP2 (GGG-ATK-GTY-CCR-AAG-CA)<br>Probe (ACA-SRA-TCT-AYA-TGC-AYC-CTG-C) |

| Clade | Molecular assay combinations | Primer and probe names (5' → 3' sequence) |
|---|---|---|
| All | Real-time RT-PCR | Fwd CCRealP1 (TCT-TYG-CHG-ATG-AYT-CHT-TYC)<br>Rev CCRealP2 (GGG-ATK-GTY-CCR-AAG-CA)<br>Probe (ACA-SRA-TCT-AYA-TGC-AYC-CTG-C) |
| | Nested RT-PCR | Fwd CrCon1+ (RWA-AYG-GRC-TTR-TGG-AYA-CYT-TCA-C)<br>Rev CrCon1– (TRG-CAA-GRC-CKG-TWG-CRA-CWA-GWG-C)<br>Rev Fwd CriCon2+ (ART-GGA-GRA-ARG-AYA-TWG-GYT-TYC-G)<br>Nested Rev CriCon2– (CYT-TGA-YRA-AYT-CYC-TRC-ACC-ABT-C) |
| | Real-time RT-PCR | Fwd CCHF-SF2 (GGA-VTG-GTG-VAG-GGA-RTT-TG)<br>Rev CCHF-SR2 (CAD-GGT-GGR-TTG-AAR-GC)<br>Probe CCHF-N2 (CAA-RGG-CAA-RTA-CAT-MAT) |
| | RT-PCR | Fwd CCS (ATG-CAG-GAA-CCA-TTA-ART-CTT-GGG-A)<br>Rev 1 CCAS1 (CTA-ATC-ATA-TCT-GAC-AAC-ATT-TC)<br>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)<br>Rev CC1a_rev (GTG-CCA-CTG-ATG-ATG-CAC-AAA-AGG-ATT-CCA-TCT)<br>Probe CCHF-01 (CAA-CAG-GCT-GCT-CTC-AAG-TGG-AG) |

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

## 167 2. Serological tests

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

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

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

182 IgM antibodies in livestock (sheep, goat and cattle) can be detected by using an IgM-capture ELISA. IgG antibodies can
183 be detected by an IgG-sandwich or indirect ELISA, and total antibodies can be detected by competition ELISA. The benefit
184 of competitive ELISA is the capacity to investigate different animal species, because they are host species independent.
185 Commercial kits for the detection of CCHFV-specific antibodies or the detection of viral antigen are available. The limiting
186 factor for the replication of these protocols in other laboratories is the availability of antigens and (where relevant) specified
187 monoclonal antibodies. Most of the tests described for livestock and wild animals have not undergone a formal validation
188 process (Mertens *et al*., 2013). One of the biggest challenges for such validation studies is the availability of an adequate
189 number of positive well characterised control samples.

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

## 192 C. REQUIREMENTS FOR VACCINES

193 There is no vaccine available for animals.

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287 *
288 * *

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

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

# 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 IS*61101) *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 IS*901 *and IS*1245), M. avium *subsp.* hominissuis (*serotypes ~~4~~–6, 8–11, and 21; lacking gene segment IS*901 *and containing segment IS*1245) *and* M. intracellulare (*serotypes 7, 12–20, and 22–28; lacking both IS*901 *and IS*1245) *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 IS*6110, *IS*901, *and IS*1245 *~~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.*

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

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

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

## A. INTRODUCTION

Several mycobacterial species can be involved in the aetiology of avian tuberculosis ~~and~~, also known as avian mycobacteriosis. Avian tuberculosis is most commonly ~~produced~~ caused by infection with *Mycobacterium avium* subsp. *avium* (serotypes 1, 2, and 3: containing specific gene segment IS*901* and nonspecific segment IS*1245*) and less frequently by *M. genavense* (Guerrero *et al*., 1995; Pavlik *et al*., 2000; Salamatian *et al.,* 2020; Sattar *et al.,* 2021; Tell *et al*., 2001). Avian mycobacteriosis is also caused by other two members of the *M. avium* complex: *M. avium* subsp. *hominissuis* (serotypes 4–6, 8–11, and 21: lacking gene segment IS*901* and containing segment IS*1245* and mainly infecting humans and pigs) and *M. intracellulare* (serotypes 7, 12–20, and 22–28: lacking both gene segments IS*901* and IS*1245*) and by ~~*M. intracellulare*, *M. scrofulaceum*, *M. fortuitum*, and~~ other potentially pathogenic mycobacterial species including *M. scrofulaceum* and *M. fortuitum*. Under some circumstances, an extensive range of mammalian species, such as swine, cattle, deer, sheep, goats, horses, cats, dogs, and exotic animals, can be infected by these mycobacterium species (Dvorska *et al*., 2004; Kunze *et al.,* 1992; Mijs *et al*., 2002; Shitaye *et al*., 2009; Tell *et al*., 2001; Thorel *et al*., 1997; 2001). *Mycobacterium tuberculosis* and *M. bovis* are ~~less common as causal~~ rarely the causative agents of tuberculosis in birds (Hoop, 2002; Lanteri *et al.,* 2011; Peters *et al.,* 2007; Schmidt *et al.,* 2022; Tell *et al*., 2001).

*Mycobacterium avium* species with standing in nomenclature as of 2023[1] (Arahal *et al.,* 2023) consists of ~~four~~ three subspecies: *M. avium* subsp. *avium*, ~~*M. avium* subsp. *hominissuis*,~~ *M. avium* subsp. *silvaticum*, and *M. avium* subsp. *paratuberculosis* (Mijs *et al*., 2002; Thorel *et al*., 1990). The latter is the causal agent of Johne's disease, or paratuberculosis, in ruminants and other mammalian species (see Chapter 3.1.16 *Paratuberculosis* [*Johne's disease*]). *Mycobacterium a. silvaticum,* which like *M. avium* subsp. *paratuberculosis* grows *in-vitro* only on media with Mycobactin, which can cause avian tuberculosis in wood pigeons (Thorel *et al*., 1990). With the widespread use of whole genome sequencing (WGS) and bioinformatics, some studies have investigated the classification of species belonging to the genus *Mycobacterium* and have proposed that *M. avium* comprises three subspecies *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis*, and *M. avium* subsp. *lepraemurium*. Further subdividing *M. avium* subsp. *avium* into three variants *M. avium* subsp. *avium* var. *avium*, *M. avium* subsp. *avium* var. *silvaticum*, and *M. avium* subsp. *avium* var. *hominissuis* (Riojas *et al.,* 2021; Tortoli *et al.,* 2019).

~~All *M. a avium* isolates from birds and mammals, including humans, have a multiple repetitive sequence IS*901* in their genome and produce a characteristic three-band pattern in IS*1245* restriction fragment length polymorphism (RFLP) as described and standardised previously (Dvorska *et al*., 2003; Ritacco *et al*., 1998). This repetitive sequence is also present in *M. a. silvaticum* and RFLP analysis can help with identification. IS*901* has only been detected in *M. avium* strains with serotypes 1, 2 and 3 (Pavlik *et al*., 2000; Ritacco *et al*., 1998) that are apparently more pathogenic to birds than other serotypes (Tell *et al*., 2001). On the basis of genetic and phenotypic differences it has recently been proposed to differentiate *M. a. avium* into two subspecies based on the target organism: *M. a. hominissuis* for human and porcine isolates and *M. a. avium* for bird-type isolates (Mijs *et al*., 2002). *Mycobacterium a. hominissuis* has polymorphic multiband IS*1245* RFLP patterns and is able to grow between 24 and 45°C (Mijs *et al*., 2002; Van Soolingen *et al*., 1998). It is worth noting that the typical features of bird-isolates, the three-band pattern in IS*1245* RFLP and presence of IS*901*, have also been found in cervine and bovine isolates of *M. a. avium*.~~

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

[1] https://lpsn.dsmz.de/species/mycobacterium-avium

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

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

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

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

It is essential to bear in mind that all members of *M. avium* ~~complex~~ and *M. genavense* are capable of giving rise to a progressive disease in humans that is refractory to treatment, especially in immunocompromised individuals (Narsana *et al.,* 2023; Pavlik *et al*., 2000; Tell *et al*., 2001). ~~Members of *Mycobacterium avium* complex are classed in Risk Group 2 for human infection and should be handled with appropriate measures~~ All *Mycobacterium* species can cause infection in people (Cowman *et al.,* 2019). Caution should be exercised by those working with birds in environments infected with *Mycobacterium*, especially those immunosuppressed. All laboratory manipulations with live cultures or potentially infected/contaminated material must be performed at an appropriate biosafety and containment level determined by conducting a thorough risk assessment as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*. ~~Biocontainment measures should be determined by risk analysis as described in Chapter 1.1.4.~~ The CDC's online Manual for Biosafety in Microbiological and Biomedical Laboratories is also a good reference[2].

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

# B. DIAGNOSTIC TECHNIQUES

***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 | ++ | +++ | + | – | ++ | – |

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction.
[(a)]A combination of agent identification methods applied to the same clinical sample is recommended.

## 1. Identification of the agent

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

### 1.1. Culture

If there is a characteristic flock history and suggestive lesions are found at necropsy, but no AFB are seen in smears or sections, an attempt must be made to isolate the causative organism from the necropsy material. The liver or spleen is usually the best organ to use, but if the carcass is decomposed, bone marrow may prove more satisfactory as it could be less contaminated. As with the culture of *M. bovis*, non-sterile specimens need to be processed with detergent, alkali, or acid to eliminate rapidly growing microorganisms before culture (see Chapter 3.1.13 *Mammalian tuberculosis* [*infection with* Mycobacterium tuberculosis *complex*]). *Mycobacterium a. avium* grows best on media such as Lowenstein–Jensen, Herrold's medium, Middlebrook 7H10 ~~and~~, 7H11, or Coletsos, with 1% sodium pyruvate added. It ~~may occasionally be~~ is necessary to incorporate mycobactin J, as it is used ~~for the isolation of~~ to isolate *M.* ~~*a. paratuberculosis*~~ *genavense* and *M. a. silvaticum.* ~~Growth may be confined to the edge of the condensation water.~~ Cultures should be incubated for at least ~~8~~ 12 weeks, less if using liquid media. Typically, *M. a. avium* produces 'smooth' colonies within 2–4 weeks; rough variants ~~do~~

occur. Shorter incubation times can be achieved using the liquid culture ~~BACTEC~~ system or the automated fluorescent ~~MGIT 960~~ culture system. *Mycobacterium a. avium* can also be detected in ~~massively~~ infected tissue by a conventional PCR, which also ~~allows acceleration of the~~ accelerates pathogen detection and identification (Moravkova *et al.,* 2008). ~~Currently,~~ Direct detection and quantification of *M. a. avium* using IS*901* quantitative real-time PCR can be considered ~~as~~ the ~~best~~ fast and inexpensive method ~~(despite its rather high cost per test)~~ (Kaevska *et al.,* 2010; Slana *et al.,* 2010).

For *M. genavense*, the optimal medium is liquid media supplemented with Mycobactin J (an iron chelator) and then plated onto a solid medium ~~is~~ such as Middlebrook 7H11 ~~medium acidified to pH 6 and supplemented with blood and charcoal (Realini *et al.*, 1999)~~. The incubation period at 37°C with 5–7% $CO_2$ should be extended for at least ~~6 months~~ 42 days. If samples are directly plated onto solid media, plates should be held for at least 12 weeks. Bacterial growth should be prepared in a smear and stained using an acid-fast stain. All acid-fast organisms should be identified using MALDI-TOF (matrix assisted laser desorption ionisation–time of flight [mass spectrometry]) or PCR (Buckwalter *et al.,* 2016; Hall *et al.,* 2003; Shitaye *et al.*, 2010).

Typing of mycobacteria to the species and subspecies level requires a specialised laboratory. Conventional biochemical tests for species identification are lengthy and fail to distinguish between *M. avium* and *M. intracellulare*. Thus, a miscellaneous group of mycobacteria that includes both species is usually classified under the ~~denomination of~~ *M. avium* complex denomination. Seroagglutination, ~~which is~~ based on the sugar residue specificity of surface glycopeptidolipids, allows ~~classification~~ the parsing of *M. avium* complex organisms into 28 serovars (Wolinsky & Schaefer, 1973). More sophisticated typing methods directed at cell-wall-specific targets are currently available, such as enzyme-linked immunosorbent assays with monoclonal antibodies to major serovars, ~~and~~ high-performance liquid chromatography ~~(HPLC)~~, and WGS. Based on DNA–rRNA hybridisation serovars 1 to 6, 8 to 11, and 21 ~~are currently~~ have been ascribed to *M. a. avium* and *M. a. hominissuis*, and serovars 7, 12 to 20, and 25 to *M. intracellulare*. However, no consensus was achieved on other serovars, and some isolates cannot be serotyped (Inderlied *et al*., 1993). For final species and subspecies identification, the current methods are WGS and bioinformatic analysis of isolates obtained from sick birds. Avian tuberculosis in birds is commonly caused by *M. a. avium* types 1, 2, or 3. If the isolate is not one of these three serotypes, further molecular identification tests ~~(IS*901* PCR)~~ must be ~~carried out~~ conducted in a specialised laboratory. However, it should be ~~borne in mind~~ noted that ~~superficial~~ tuberculous lesions in ~~caged~~ ~~pet~~ captive birds, especially psittacines, may be caused by *M. tuberculosis,* and ~~IS*6110* PCR should be used for~~ precise identification should always be attempted (Hoop, 2002; Lanteri *et al*., 2011; Peters *et al*., 2007; Schmidt *et al*., 2008; Tell *et al*., 2001).

## 1.2. Nucleic acid recognition methods

Specific and reliable genetic tests for speciation ~~are currently~~ have been available (Saito *et al*., 1990)~~.~~, including commercial nucleic ~~acid~~ hybridisation probes ~~have become a 'gold standard'~~ reference method for ~~distinction between~~ distinguishing *M. avium*, ~~and~~ *M. intracellulare* ~~cultures.~~ and *M. genavense* ~~can also be distinguished with these tests~~. A further probe that covers the whole *M. avium* complex was also developed, as genuine *M. avium* complex strains have been described that fail to react with specific *M. avium* and *M. intracellular* probes (Soini *et al*., 1996). Nevertheless, identification errors were reported due to the cross-reactivity, which may have serious consequences (van Ingen *et al.,* 2009). Various in-house molecular methods have been reported ~~for the identification of~~ to identify mycobacterial cultures, including ~~MAC.~~ members of the *Mycobacterium avium* complex. The following gene segments could be used to identify *Mycobacterium* isolates as *M. avium* in one multiplex PCR reaction: IS*900*, IS*901*, IS*1245*. The isolates of *M. a. avium/M. a. silvaticum* are IS*900*−, IS*901*+, IS*1245*+, the isolates of *M. a. hominissuis* are IS*900*−, IS*901*−, IS*1245*+, and the isolates of *M. a. paratuberculosis* are IS*900*+, IS*901*−, IS*1245*− (Kaevska *et al.,* 2010; Moravkova *et al.,* 2008). A ~~multiplex~~ 16S rRNA PCR and sequencing method for differentiating *M. avium* from *M. intracellulare* and *M. tuberculosis* complex ~~has some advantages (Cousins *et al*., 1996). 16S rRNA~~ is currently commercially available. Similarly, many veterinary diagnostic laboratories commonly perform in-house PCR and sequencing (Kirschner *et al*., 1993) ~~may also be used~~. Culture-independent in-house molecular tests have been developed ~~for the detection~~ to detect and ~~identification of~~ identify species belonging to the *M. avium* complex directly from samples (Hall *et al.,* 2003; Kaevska *et al.,* 2010). WGS of isolates has recently become the go-to molecular method to identify mycobacterium isolates from birds with great accuracy. It enables, with the use of bioinformatic tools, not only an accurate identification of species and subspecies, but also helps to determine the organism relatedness within a flock or environment (Witte *et al.,* 2021). In recent years, veterinary diagnostic laboratories have extensively adopted real-time PCR methods to detect *M. a. avium* directly from different specimens (faeces, tissues, formalin-fixed tissues, and environmental samples). The technique rapidly detects fastidious and slow-growing microorganisms, such as *M. a. avium* (Tell *et al.,* 2003a; 2003b).

Several commercial diagnostic PCR tests for detecting *M. a. avium* are available. Still, users should consider the skill set and equipment necessary to perform such tests. Furthermore, it is important to determine the fitness

for the purpose of these tests before implementation. The interpretation of the results of these molecular tests also requires veterinary expertise.

~~*Mycobacterium a. avium,* the causative agent of avian tuberculosis (Thorel *et al.,* 1990), previously designated as *M. avium* species only, is assigned to serotypes 1 to 3 within the *M. avium* complex of 28 serotypes (Wolinsky & Schaefer, 1973). As revealed by molecular biological studies, the detected insertion sequence IS*901* (Kunze *et al.,* 1992) is possessed not only by the isolates of the above-named serotypes, but also by isolates, virulent for birds, that could not be typed because agglutination occurred (Pavlik *et al.,* 2000). In epidemiological studies, a standardised IS*901* RFLP methods replaced serotyping (Dvorska *et al.,* 2003).~~

## 2. Immunological methods

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

### 2.1. Tuberculin test

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

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

### 2.2. Stained antigen test

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

#### 2.2.1. Preparation of the antigen

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

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

desired properties of sensitivity and specificity. Consistency of results between batches will be easier using pure cultures.

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

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

The bottles are incubated at 37°C, and good growth should be obtained in 14–21 days with most strains. The antigen is harvested by adding sterile glass beads and twice the volume of sterile normal saline (containing 0.3% formalin) as was used to inoculate the bottle. The bottle is then shaken gently to wash off all the growth, and the washing is collected into a sterile bottle and re-incubated at 37°C for 7 days. The killed bacilli are washed twice in sterile normal saline with 0.2% formalin by centrifugation and re-suspension. This sequence is safer than the original method in which the washing was carried out before the incubation that kills the organisms. Finally, bacilli are again centrifuged and re-suspended in sterile normal saline containing 0.2% formalin and 0.4% sodium citrate to a concentration of about $10^{10}$ bacteria per ml. This corresponds to a concentration ten times that which matches tube No. 4 on McFarland's scale.

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

#### 2.2.2. Validation of the antigen

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

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

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

## Note on limitation of use

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

# C. REQUIREMENTS FOR DIAGNOSTIC BIOLOGICALS

## 1. Background

No vaccines are available.

Avian tuberculin is a preparation of purified protein derivatives (PPD-A) made from the heat-treated products of growth of *M. a. avium*. It is used by intradermal injection to reveal delayed hypersensitivity ~~as a means of identifying~~ to identify birds infected with or sensitised to the same species of ~~tubercle bacillus~~ *Mycobacterium*. Importantly it is also used ~~as an~~ to aid ~~to~~ differential diagnosis in the comparative intradermal tuberculin test for bovine tuberculosis (see Chapter 3.1.13). An international standard preparation of PPD-A is being developed by WOAH to replace the former WHO Standard[3].

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

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

### 2.1. Characteristics of the seed

#### 2.1.1. Biological characteristics of the master seed

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

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

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

### 2.2. Method of manufacture

#### 2.2.1. Procedure

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

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

#### 2.2.2. Requirements for ingredients

The production culture substrate must be shown to ~~be capable of producing~~ produce a product that conforms to the ~~standards of the~~ European Pharmacopoeia (~~2000~~ 2024[4]) standards or other international standards such the WHO (WHO, 1987). It must be free from ingredients known to cause toxic or allergic reactions.

#### 2.2.3. In-process controls

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

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

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

### 2.2.4. Final product batch tests

i) Sterility

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

ii) Identity

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

iii) Safety

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

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

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

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

iv) Batch potency

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

Use no fewer than nine albino guinea-pigs, each weighing 400–600 g. Sensitise the guinea-pigs by administering ~~to each, by deep intramuscular injection,~~ a suitable dose of inactivated or live *M. a. avium* to each by deep intramuscular injection. The test is performed between 4 and 6 weeks later ~~as follows: Shave~~. Briefly, have the guinea-pigs' flanks shaved (an area large enough ~~so as~~ to provide space for three-to-four injections on each side). Prepare at least three dilutions of the tuberculin under test and at least three dilutions of the standard preparation in an isotonic buffer solution containing 0.0005% (w/v) polysorbate 80 (Tween 80). Choose the dilutions so that the reactions produced have diameters of not less than 8 mm and not more than 25 mm. Allocate the

dilutions to the injection sites randomly ~~according to~~ using a Latin square design. The dilutions correspond to 0.001, 0.0002, and 0.00004 mg of protein in a final dose of 0.2 ml, injected intradermally.

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

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

## 3. Requirements for authorisation/registration/licensing

### 3.1. Manufacturing process

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

### 3.2. Safety requirements

#### 3.2.1. Target and non-target animal safety

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

#### 3.2.2. Precautions (hazards)

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

### 3.3. Stability

During storage, liquid avian tuberculin should be protected from the light and held at a temperature of 5°C (±3°C). Freeze-dried preparations may be stored at higher temperatures (~~but~~ not exceeding 25°C) and protected from ~~the~~ light. During use, periods of exposure to higher temperatures or to direct sunlight should be kept at a minimum.

~~Provided the tuberculins are~~ Following accepted practice, tuberculin should be stored at a temperature of between 2°C and 8°C and protected from light; they may be used up to the end of the following periods ~~subsequent to~~ after the last satisfactory potency test: Liquid PPD tuberculin~~s~~: 2 years; lyophilised PPD-A tuberculin~~s~~: 8 years; HCSM (heat-concentrated synthetic-medium) tuberculin~~s~~ diluted: 2 years. Recent research on the temperature stability of human, bovine, and avian tuberculin solutions has shown that they are stable for a year at 37°C. This should be further explored as these products are used in the field in remote areas of the world where maintaining temperature control is very difficult (Maes *et al.*, 2011).

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618 * *

619 **NB:** There is currently (2024) no WOAH Reference Laboratory for avian tuberculosis
620 (please consult the WOAH Web site for the current list:
621 https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3http://www.oie.int/).

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

**Annex 10. Item 5.1. – Chapter 3.4.1. Bovine anaplasmosis**

**MEETING OF THE BIOLOGICAL STANDARDS COMMISSION**

**Paris, 5–9 February 2024**

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# S E C T I O N 3 . 4 .

**B O V I N A E**

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## C H A P T E R 3 . 4 . 1 .

# B O V I N E A N A P L A S M O S I S

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

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

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

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

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

# A. INTRODUCTION

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

The most marked clinical signs of bovine anaplasmosis are anaemia and jaundice, the latter occurring in acute severe, cases or late in the disease. Haemoglobinaemia and haemoglobinuria are not present, and this may assist in the differential diagnosis of bovine anaplasmosis from babesiosis, which is often endemic in the same regions. The disease can only be confirmed, however, by identification of the organism in erythrocytes from the affected animal. Caution must be exercised if using nucleic acid techniques alone to diagnose *A. marginale* in anaemic cattle. Persistent, low-level infection can be detected by these techniques and may lead to a misdiagnosis of bovine anaplasmosis. Visualisation of *A. marginale* bodies in erythrocytes is therefore required for confirmation.

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

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

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

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

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

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

*Anaplasma marginale* infection has not been reported in humans. ~~Thus,~~ There is ~~no~~ minimal risk of field or laboratory transmission to workers ~~and~~ from laboratories working with *A. marginale* ~~may operate at the lowest biosafety level, equivalent to BSL1~~. Nevertheless the agent should be handled with appropriate biosafety and containment procedures as determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

## B. DIAGNOSTIC TECHNIQUES

***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 | – | – | – | – | – | ++ |

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
Agent id. = agent identification; CAT = card agglutination test; ~~CFT = complement fixation test;~~
C-ELISA = competitive enzyme-linked immunosorbent assay; ddasELISA = displacement double-antigen, sandwich ELISA;
IFAT = indirect fluorescent antibody test; PCR = polymerase chain reaction.
[(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)]See Appendix 3 of this chapter for justification table for the scores giving to the tests for this purpose.
[(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.
[(h)]These tests do not distinguish infected from vaccinated animals.

## 1. Detection of the agent

### 1.1. Microscopic examination

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

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

the diagnosis of anaplasmosis, as ~~*Anaplasma*~~ *A. marginale* are difficult to identify once they become dissociated from erythrocytes.

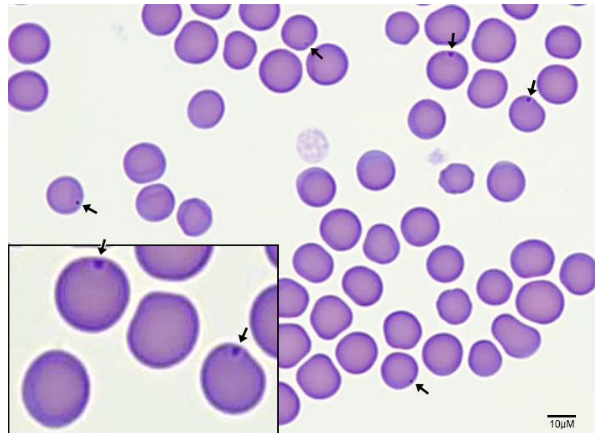


Fig. 1. *Anaplasma marginale* ~~initial~~ inclusion bodies. A ~~Diff-Quick~~ stained blood smear from a bovine experimentally infected with *A. marginale*. Arrows point to the *A. marginale* ~~initial~~ inclusion bodies. Photo from S. Noh.

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

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

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

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

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

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

### 1.2. Polymerase chain reaction

Nucleic acid-based tests to detect *A. marginale* ~~infection~~ in ~~carrier~~ infected cattle have been developed although not ~~yet~~ fully validated. The analytical sensitivity of polymerase chain reaction (PCR)-based methods has been estimated at 0.0001% infected erythrocytes, but at this level, only a proportion of

carrier cattle would be detected. A nested PCR has been used to identify *A. marginale* carrier cattle with a capability of identifying as few as 30 infected erythrocytes per ml of blood, well below the lowest levels in carriers. However, nested PCR is time consuming as it requires two full PCR reactions, and poses significant quality control ~~and specificity~~ problems for routine use (Torioni De Echaide *et al.*, 1998). Real-time PCR assays are reported to achieve a level of analytical sensitivity equivalent to nested PCR ~~has also been described for identification of *A. marginale*~~ and should be considered instead of the nested PCR (Carelli *et al.*, 2007; Decaro *et al.*, 2008~~; Reinbold *et al.*, 2010b~~). ~~Two~~ Advantages of ~~this technique~~ the real-time PCR, which uses a single closed tube for amplification and analysis, are reduced ~~opportunity for~~ risk of amplicon contamination and a semi-quantitative assay result. Equipment and reagents needed for real-time PCR ~~is~~ are expensive~~, requires preventive maintenance,~~ and may be beyond the capabilities of some laboratories. ~~Real-time PCR assays may target one of several genes (Carelli *et al.*, 2007; Decaro *et al.*, 2008), or 16S rRNA (Reinbold *et al.*, 2010b), and are reported to achieve a level of analytical sensitivity equivalent to nested conventional PCR (Carelli *et al.*, 2007; Decaro *et al.*, 2008; Reinbold *et al.*, 2010b).~~

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

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

*Msp5* has also been used as a target to detect *A. marginale* in cattle in field samples and more frequently in experimental samples (Futse *et al.*, 2003). *Msp5* is highly conserved among *A. marginale* strains and is a single copy gene, thus providing some advantages as a target for ensuring detection of widely variant strains of *A. marginale*. However, the related *Anaplasma* spp. and *Ehrlichia* spp. all have *msp5* orthologs with 50% identity to an *E. ruminantium* gene (NCBI accession: L07385.1), thus specificity must be determined in laboratory and field samples. Additionally, little work has been done to validate an *msp5*-based real-time PCR test for diagnostic purposes.

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

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

261 ***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 *et al.*, 2007 | *Am_msp1b*_F | TTG-GCA-AGG-CAG-CAG-CTT | 95 | M59845 |
| | | *Am_msp1b*_R | TTC-CGC-GAG-CAT-GTG-CAT | | |
| | | *Am_msp1b*_PB | TCG-GTC-TAA-CAT-CTC-CAG-GCT-TTC-AT | | |
| Real-time PCR | Futse *et al.*, 2003 | *Am_msp5*_F | GCC-AAG-TGA-TGG-TGA-TAT-CGA | 151 | M93392 |
| | | *Am_msp5*_R | AGA-ATT-AAG-CAT-GTG-ACC-GCT-G | | |
| | | *Am_msp5*_PB | AAC-GTT-CAT-GTA-CCT-CAT-CAA | | |
| Reverse-transcription real-time PCR | Reinbold *et al.*, 2010 | *16S rRNA*_F[c] | CTC-AGA-ACG-AAC-GCT-GG | 142 | M60313 |
| | | *16S rRNA*_R[c] | CAT-TTC-TAG-TGG-CTA-TCC-C | | |
| | | *Am_16S rRNA*_PB[d] | CGC-AGC-TTG-CTG-CGT-GTA-TGG-T | | |
| Real-time PCR[d] | Decaro *et al.*, 2008 | *Ac_groEL*_F[e, f] | CTA-TAC-ACG-CTT-GCA-TCT-C | 77 | CP001759.1 |
| | | *Ac_groEL*_R[e, f] | CGC-TTT-ATG-ATG-TTG-ATG-C | | |
| | | *Ac_groEL*_PB[e, f] | TCA-TCA-TTC-TTC-CCC-TTT-ACC-TCG-T | | |

262 [a]*Am* denotes *A. marginale*, *Ac* denotes *A. centrale*, Pb denotes probe sequence.
263 [b]Fluorophores and quenchers not included in probe sequences.
264 [c]Amplifies *A. phagocytophilum* and *A. marginale* 16S rRNA gene.
265 [d]Probe is specific for *A. marginale* 16S rRNA gene.
266 [e]Can be used as a duplex PCR with *msp1b* primers and probe based on Carelli *et al.*, 2007.
267 [f]Primers and probe amplify *A. centrale groEL*.

268 ## 2. Serological tests

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

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

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

293 ### 2.1. Competitive enzyme-linked immunosorbent assay

294 A C-ELISA using a recombinant antigen termed Major surface protein 5 (MSP5) is an immunodominant
295 protein expressed by A. *marginale*, *A. ovis*, and *A. centrale*. In *A. marginale* the gene is highly conserved
296 making it a useful target across broad geographical regions with high *A. marginale* strain diversity

(Knowles *et al.*, 1996; Torioni De Echaide *et al.*, 1998). Thus, a C-ELISA based on recombinantly expressed (rMSP5~~ and MSP5~~) in combination with an MSP5-specific monoclonal antibody (mAb) has proven very sensitive and specific for detection of *Anaplasma*-infected animals (~~Hofmann-Lehmann *et al.*, 2004~~ Molloy *et al.*, 1999; Reinbold *et al.*, 2010b; Strik *et al.*, 2007). ~~All *A. marginale* strains tested, along with~~ Additionally, A. *ovis* and *A. centrale*, express ~~the~~ MSP5 ~~antigen~~ and ~~induce~~ infected animals produce antibodies against the immunodominant epitope recognised by the MSP5-specific ~~mAb. A recent report~~ mAb used in the C-ELISA. This C-ELISA was updated in 2014 to improve performance by using glutathione S-transferase (GST) instead of maltose binding protein (MBP) as the tag on the rMSP5 (Chung *et al.*, 2014). This assay no longer requires adsorption to remove the antibodies directed against MBP, thus it is faster and easier than the previous version of the C-ELISA. The diagnostic sensitivity is 100% and the diagnostic specificity is 99.7% using a cut-off of 30% inhibition as determined by receiver operating characteristic (ROC) plot (Chung *et al.*, 2014). For this validation, 385 sera defined as negative were from dairy cattle maintained in tick-free facilities from farms with no clinical history of bovine anaplasmosis. The 135 positive sera were from cattle positive for *A. marginale* using nested PCR and serology.

One study suggested that antibodies from cattle experimentally infected with *A. phagocytophilum* will test positive in the C-ELISA (Dreher *et al.*, 2005). However, in another study no cross-reactivity could be demonstrated, and the mAb used in the assay did not react with *A. phagocytophilum* MSP5 in direct binding assays (Strik *et al.*, 2007). Cross reactivity has been demonstrated between ~~*A. marginale* and *Ehrlichia* spp, in naturally and experimentally infected cattle (Al-Adhami *et al*, 2011). Earlier studies had shown that the C-ELISA was 100% specific using 261 known negative sera from a non-endemic region, detecting acutely infected cattle as early as 16 days after experimental tick or blood inoculation, and was demonstrated to detect cattle that have been experimentally infected as long as 6 years previously (Knowles *et al.*, 1996). In detecting persistently infected cattle from an anaplasmosis-endemic region that were defined as true positive or negative using a nested PCR procedure, the rMSP5 C-ELISA had a sensitivity of 96% and a specificity of 95% (Torioni De Echaide *et al.*, 1998)~~ *A. marginale* and *Ehrlichia* sp. BOV2010 isolated in Canada, in naturally and experimentally infected cattle (Al-Adhami *et al*, 2011).

Test results using the rMSP5 C-ELISA are available in less than 2~~.5~~ hours. A test kit is available commercially that contains specific instructions. Users should follow the manufacturer's instructions. ~~In general, however, it is conducted as follows.~~

### ~~2.1.1. Kit reagents~~

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

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

~~100×Mab-peroxidase conjugate,~~

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

~~Ready-to-use substrate and stop solutions,~~

~~Positive and negative controls~~

### ~~2.1.2. Test procedure~~

~~i) Add 70 µl of undiluted serum sample to the coated adsorption/transfer plate and incubate at room temperature for 30 minutes.~~

~~ii) Transfer 50 µl per well of the adsorbed undiluted serum to the rMSP5-coated plate and incubate at room temperature for 60 minutes.~~

~~ii) Discard the serum and wash the plate twice using diluted wash solution.~~

~~iii) Add 50 µl per well of the 1× diluted MAb-peroxidase conjugate to the rMSP5-coated plate wells, and incubate at room temperature for 20 minutes.~~

~~iv) Discard the 1×diluted MAb-peroxidase conjugate and wash the plate four times using diluted wash solution.~~

~~v) Add 50 µl per well of the substrate solution, cover the plate with foil, and incubate for 20 minutes at room temperature.~~

~~vi) Add 50 µl per well of stop solution to the substrate solution already in the wells and gently tap the sides of the plate to mix the wells.~~

~~vii) Immediately read the plate in the plate reader at 620, 630 or 650 nm.~~

349 #### 2.1.3. Test validation

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

352 #### 2.1.4. Interpretation of the results

353 The % inhibition is calculated as follows:

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

354 % inhibition = 100[1 – (Sample OD ÷ Negative Control OD)]

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

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

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

363 ### 2.2. Indirect enzyme-linked immunosorbent assay

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

378 Alternatively, rMSP5 can be used as the antigen in this test. This eliminates the need for preparation and
379 standardisation of antigen derived from splenectomised, *A. marginale* infected animals (Silva *et al*.,
380 2006). In a comparison between I-ELISA using the CAT antigen and rMSP with a histidine tag (rMSP5-
381 HIS), these two I-ELISAs performed identically. In this comparison, IFAT was used as the gold standard
382 test (Silva *et al*., 2006).

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

384 #### 2.2.1. Test reagents

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

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

387 Blocking reagent (e.g. commercial dried skim milk)

388 Tris buffer 0.1 M, $MgCl_2$, 0.1 M, NaCl, 005 M, pH 9.8

389 Substrate *p*-Nitrophenyl phosphate disodium hexahydrate

390 Positive and negative controls.

391 #### 2.2.2. Test procedure (this test is run in triplicate)

392 i) Plates can be prepared ahead of time and kept under airtight conditions at –20°C.

ii) Carefully remove the plastic packaging before using plates, being careful not to touch the bottom of them as this can distort the optical density reading.

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

iv) For one plate, dissolve 1.1 g of skim milk (blocking agent) in 22 ml of PBST20.

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

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

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

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

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

x) Wash three times as described in point ~~subsection~~ vi.

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

xii) Remove the lid and wash three times as described in point vi above ~~make three washes with PBST20~~.

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

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

### 2.2.3. Data analysis

Analysis of results should take into account the following parameters.

i) The mean value of the blank wells.

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

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

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

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

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

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

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

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

In regions where vaccination with *A. centrale* is used to control bovine anaplasmosis, differentiation between *A. centrale*-vaccinated and *A. marginale*-infected animals may be useful. Because there is often high amino acid identity between *A. marginale* and *A. centrale* surface proteins, identifying unique targets for serological assays for this purpose is difficult. Epitopes from MSP5 (aa28-210, without the transmembrane region) that are not shared between *A. marginale* and *A. centrale* were used to develop a displacement double-antigen sandwich ELISA (ddasELISA) (Bellezze *et al*., 2023; Sarli *et al*., 2020). The recombinant MSP5 epitopes from *A. marginale* or *A. centrale* are expressed in *E. coli* with a histidine tag and purified. The ELISA plates are then coated with either the recombinant *A. marginale* MSP5 epitope, or the *A. centrale* MSP5 epitope and blocked. Serum is added to the wells and allowed to incubate. Following washing, a combination of biotinylated and non-biotinylated recombinant proteins

442 are added to improve specificity of the reaction (see below for specifics). The protein–biotin binding to
443 the serum antibody is detected with a peroxidase-streptavidin based detection system. The optical
444 density for the *A. marginale* MSP5-coated well (ODAm) and the OD for the *A. centrale* MSP5 (ODAc)
445 coated well for each animal is measured. If the OD for either target is <0.2, the sample is excluded from
446 the analysis. For the remaining samples, the ratio between the OD values (ODAm/ODAc) is calculated.
447 If the ratio is >0.38 the sample is considered positive for anti-*A. marginale* antibodies, and a ratio ≤ 0.38
448 is classified as vaccinated with *A. centrale*.

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

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

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

463 **2.3.1. Test reagents**

464 i) A 96-well microtitre plate coated with either *A. marginale* or A. centrale recombinant protein

465 ii) PBS/Tween buffer (PBS (50mM sodium phosphate, 150 mM NaCL, pH 7.2) with 0.05%
466 Tween-20)

467 iii) Blocking reagent (PBS with 10% commercial dried skim milk)

468 iv) Purified recombinant *A. marginale* MSP5 epitopes and *A. centrale* epitopes

469 v) Biotinylated recombinant *A. marginale* MSP5 epitopes and *A. centrale* epitopes

470 vi) Streptavidin-horse radish peroxidase (HRP) detection system

471 vii) Chromogenic substrate (1 mM 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-
472 diammonium salt in0.05 M sodium citrate, pH 4.5, 0.0025% V/V $H_2O_2$ (100 µl/well).

473 viii) ELISA plate reader (405 nm reading)

474 ix) Positive and negative control sera for *A. marginale* and *A. centrale*

475 **2.3.2. Test procedure**

476 i) Plates are coated overnight.

477 ii) Block with blocking buffer for 1 hour at room temperature and wash three times with
478 PBS/Tween buffer.

479 iii) Add undiluted serum 100 µl/well and incubate at 25°C for 1 hour at 100 rpm.

480 iv) Wash three times with PBS/Tween buffer.

481 v) Add 100 µl of *A. marginale* MSP5-biotin (1 µg/ml) plus *A. centrale* MSP5 (10 µg/ml) to
482 *A. marginale* test wells. Add *A. centrale* MSP5-biotin (1 µg/ml) plus *A. marginale* MSP5
483 (10 µg/ml) in PBS/Tween buffer + 10% fat-free dried milk to *A. centrale* test wells.

484 vi) Incubate at 25°C for 1 hour at 100 rpm and wash the plate five times with PBS/Tween buffer.

485 vii) To detect the bound protein–biotin complex, add streptavidin-HRP diluted in 1/500 in
486 PBS/Tween buffer with 10% dried milk for 1 hour at 25°C, 100 rpm.

487 vii) Wash five times with PBS/Tween buffer.

488 ix) Add chromogenic substrate based on manufacturer's instructions.

489 x) The reaction is measured by microplate reader spectrophotometer at 405 nm wavelength.
490 The data are expressed in optical density (OD).

491 xi) $OD_{405nm}$ <0.2 is considered negative.

xii) Results are expressed as the ratio between antibodies specific for *A. marginale* MSP5 and for *A. centrale* MSP5 (ODAm/ODAc). If the ratio is >0.38 the sample is considered positive for anti-*A. marginale* antibodies, and a ratio ≤ 0.38 is classified as vaccinated with *A. centrale*.

### 2.4. Card agglutination test

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

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

#### 2.4.1. Test procedure

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

ii) On each circle of the test card (a clear perspex/plastic or glass plate marked with circles that are 18 mm in diameter), place next to each other, but not touching, 10 µl of bovine serum factor (BSF), 10 µl of test serum, and 5 µl of CAT antigen[1]. Negative and low positive control sera must be tested on each card.

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

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

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

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

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

### ~~2.4. Complement fixation test~~

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

### 2.5. Indirect fluorescent antibody test

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

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

### 2.6. Complement fixation test

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

# C. REQUIREMENTS FOR VACCINES

## 1. Background

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

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

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.

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

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

### 2.1. Characteristics of the seed

#### 2.1.1. Biological characteristics

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

*Anaplasma centrale* usually causes benign infections, especially if used in calves under 9 months of age. Severe reactions following vaccination have been reported when adult cattle are inoculated. The suitability of an isolate of *A. centrale* as a vaccine can be determined by inoculating susceptible cattle, monitoring the subsequent reactions, and then challenging the

animals and susceptible controls with a virulent local strain of *A. marginale.* Both safety and efficacy can be judged by monitoring rickettsaemias in stained blood films and the depression of packed cell volumes of inoculated cattle during the vaccination and challenge reaction periods.

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

#### 2.1.2. Quality criteria

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

### 2.2. Method of manufacture

#### 2.2.1. Procedure

**i) Production of frozen vaccine**

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

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

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

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

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

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

**ii) Production of chilled vaccine**

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

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

**iii) Use of vaccine**

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

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

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

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

### 2.2.2. Requirements for substrates and media

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

### 2.2.3. In-process controls

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

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

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

**ii) Surgery**

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

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

As for preparation of seed stabilate, donor calves for vaccine production should be examined for all blood-borne infections prevalent in the vaccine-producing country, including *Babesia*, *Anaplasma*, *Ehrlichia*, *Theileria* and *Trypanosoma*. This can be done by routine examination

698 of stained blood films after splenectomy, and preferably also by serology. Any calves
699 showing evidence of natural infections of any of these agents should be rejected. The
700 absence of other infective agents should also be confirmed. These may include the agents
701 of enzootic bovine leukosis, bovine viral diarrhoea, infectious bovine rhinotracheitis,
702 ephemeral fever, Akabane disease, bluetongue, and foot and mouth disease. The testing
703 procedures will depend on the diseases prevalent in the country and the availability of tests,
704 but should involve serology of paired sera at the very least and, in some cases, virus
705 isolation, antigen, or DNA/RNA detection (Bock *et al.*, 2004; Pipano, 1981; 1997).

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

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

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

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

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

717 **vi) Dispensing of vaccine**

718 All procedures are performed in a suitable environment, such as a laminar flow cabinet,
719 using standard sterile techniques. Use of a mechanical or magnetic stirrer will ensure
720 thorough mixing of blood and diluent throughout the dispensing process. Penicillin
721 (500,000 IU/litre) and streptomycin (370,000 µg/litre) are added to the vaccine at the time of
722 dispensing.

723 **2.2.4. Final product batch tests**

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

727 **i) Sterility and purity**

728 Standard tests for sterility are employed for each batch of vaccine and diluent (see Chapter
729 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for*
730 *veterinary use*).

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

746 **ii) Safety**

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

**iii) Potency**

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

### 2.3. Requirements for authorisation

#### 2.3.1. Safety

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

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

#### 2.3.2. Efficacy requirements

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

#### 2.3.3. Stability

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

## 3. Vaccines based on biotechnology

There are no vaccines based on biotechnology available for anaplasmosis.

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916 **NB:** There is a WOAH Reference Laboratory for anaplasmosis (please consult the WOAH Web site:
917 https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3http://www.oie.int/)
918 Please contact the WOAH Reference Laboratory for any further information on
919 diagnostic tests, reagents and vaccines for bovine anaplasmosis

920 **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

| Test with score and species | Sample type and target analytes | Accuracy | Test population | Validation report | Advantages | Disadvantages | References |
|---|---|---|---|---|---|---|---|
| C-ELISA<br>+++<br>Bovine | Serum<br>rMSP5-GST | Reference tests were nested PCR and IFAT.<br>Dsp = 99.7%<br>Dse = 100%<br>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.<br>2. 135 known positive sera as defined by nested PCR.<br>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.<br>2. High sensitivity, detects persistently infected animals.<br>3. Commercially available.<br>4. Uses a standardised antigen.<br>5. Target antigen is highly conserved among *A. marginale* strains, thus detects infection with all strains of *A. marginale*.<br>6. Rapid. | 1. Does not differentiate between infection with *A. marginale* and *A. centrale*.<br>2. May cross react with anti-*Ehrlichia* antibodies.<br>3. May not be readily available in all countries.<br>4. Requires a microplate absorbance reader.<br>5. Low percent of false positive results. | Chung *et al.*, 2014. |
| IFAT+<br>Bovine | Serum<br>Glass slides with RBCs infected with *A. marginale*. | Reference test was blood smear.<br>DSe 97.6%<br>Dsp 89.6% | 48 cattle raised in anaplasmosis free region.<br>82 animals from endemic region. | See reference | 1. Antigen is relatively easy to produce and store.<br>2. Does not require many reagents. | 1.Low specificity.<br>2. Time consuming and labour intensive so not suitable for high throughput.<br>3. Requires fluorescent microscope and blood smears with high rickettsemia. | Gonzalez *et al.*, 1978 |

3 **Appendix 2: Bovine anaplasmosis**

4 **Intended purpose of test: Individual animal freedom from infection prior to movement.**

| **Test with score and species** | **Sample type and target analytes** | **Accuracy** | **Test population** | **Validation report** | **Advantages** | **Disadvantages** | **References** |
|---|---|---|---|---|---|---|---|
| PCR ++ | Whole blood<br>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 *A. marginale, A. centrale, A. bovis, T. buffeli, B bovis, A. phagocytophilum*, and *B. bigemina*. 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 *et al.*, 2007. |
| C-ELISA +++<br>Bovine | Serum<br>rMSP5-GST | Reference tests were nested PCR and IFAT.<br>Dsp = 99.7%<br>Dse = 100%<br>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.<br>2. 135 known positive sera as defined by nested PCR.<br>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.<br>2. High sensitivity, detects persistently infected animals.<br>3. Commercially available.<br>4. Uses a standardised antigen.<br>5. Target antigen is highly conserved among *A. marginale* strains, thus detects infection with all strains of *A. marginale*.<br>6. Rapid. | 1. Does not differentiate between infection with *A. marginale* and *A. centrale*.<br>2. May cross react with anti-*Ehrlichia* antibodies.<br>3. May not be readily available in all countries.<br>4. Requires a microplate absorbance reader. | Chung *et al.*, 2014. |

5 [1.] RLB is the reverse line blot test.

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

| **Test with score and species** | **Sample type and target analytes** | **Accuracy** | **Test population** | **Validation report** | **Advantages** | **Disadvantages** | **References** |
|---|---|---|---|---|---|---|---|
| C-ELISA<br>+++<br>Bovine | Serum<br>rMSP5-GST | Reference tests were nested PCR and IFAT.<br>Dsp = 99.7%<br>Dse = 100%<br>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.<br>2. 135 known positive sera as defined by nested PCR.<br>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.<br>2. High sensitivity, detects persistently infected animals.<br>3. Commercially available.<br>4. Uses a standardised antigen.<br>5. Target antigen is highly conserved among *A. marginale* strains, thus detects infection with all strains of *A. marginale*.<br>6. Rapid. | 1. Does not differentiate between infection with *A. marginale* and *A. centrale*.<br>2. May cross react with anti-*Ehrlichia* antibodies.<br>3. May not be readily available in all countries.<br>4. Requires a microplate absorbance reader.<br>5. Low percent of false positive results. | Chung *et al*., 2014) |

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

| Test with score and species | Sample type and target analytes | Accuracy | Test population | Validation report | Advantages | Disadvantages | References |
|---|---|---|---|---|---|---|---|
| Microscopic examination +++ | Whole blood | No robust validation has been published. | N/A | N/A | 1. Most laboratories have the capacity to make and examine blood smears.<br>2. *A. marginale* infected erythrocytes readily visible in clinically affected animals. | 1. *A. marginale* colonies are small and can be difficult to differentiate from debris if animal has low rickettsemia.<br>2. Requires experience to identify *A. marginale* colonies.<br>3. Difficult to differentiate between *A. marginale* and *A. centrale*. | |
| PCR +++ | Whole blood<br>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 *A. marginale A. centrale, A. bovis, T. buffeli, B bovis, A. phagocytophilum*, and *B. bigemina*. 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). | 1. Must be performed in a lab equipped to extract DNA and have thermocyclers for real-time PCR.<br>2. Important to use PCR in conjunction with diagnosis of anaemia and blood smear because PCR can detect low level rickettsemia leading to misdiagnosis. | Carelli *et al*., 2007 |

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

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

| Test with score and species | Sample type and target analytes | Accuracy | Test population | Validation report | Advantages | Disadvantages | References |
|---|---|---|---|---|---|---|---|
| CAT<br>+ | Serum<br>Lysates of *A. marginale* isolated from red blood cells. | Reference test was blood smear.<br>DSe 84.1[1]-100[2]%<br>Dsp 97.9[1]-98.6[2]% | 48 cattle raised in anaplasmosis free region.<br>82 animals from endemic region.[1]<br>86 sera from experimentally infected cattle and 183 sera from *A. marginale* free area[2] | See references | 1. Can be done in field or in the laboratory | 1. Antigen derived from infected cattle are difficult to produce and standardise.<br>2. May have false negative and false positive results.<br>3. Variation between tests depending on environmental conditions and the laboratory. | [1]Gonzalez *et al*., 1978.<br>[2]Molloy *et al*., 1999. |
| C-ELISA<br>+++<br>Bovine | Serum<br>rMSP5-GST | Reference tests were nested PCR and IFAT.<br>Dsp = 99.7%<br>Dse = 100%<br>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.<br>2. 135 known positive sera as defined by nested PCR.<br>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.<br>2. High sensitivity, detects persistently infected animals.<br>3. Commercially available.<br>4. Uses a standardised antigen.<br>5. Target antigen is highly conserved among *A. marginale* strains, thus detects infection with all strains of *A. marginale*.<br>6. Rapid. | 1. Does not differentiate between infection with *A. marginale* and *A. centrale*.<br>2. May cross react with anti-*Ehrlichia* antibodies.<br>3. May not be readily available in all countries.<br>4. Requires a microplate absorbance reader.<br>5. Low percent of false positive results | Chung *et al*., 2014. |
| IFAT++<br>Bovine | Serum<br>Glass slides with RBCs infected with *A. marginale* | Reference test was blood.<br>DSe 97.6%<br>Dsp 89.6% | 1. 48 cattle raised in anaplasmosis free region.<br>2. 82 animals from endemic region. | See references | 1. Antigen is relatively easy to produce and store.<br>2. Does not require many reagents. | 1.Relatively high false positive rate.<br>2. Time consuming and labour intensive so not suitable for high throughput.<br>3. Requires fluorescent microscope and blood smears with high rickettsemia. | Gonzalez *et al*., 1978 |

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## Appendix 6: 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 | - Very sensitive<br>- Rapid<br>- High-throughput<br>- Well established internationally<br>- Detects assay-dependent all BVDV species<br>- Allows assay-dependent for differentiation of BVDV types 1 and 2<br>- Detects persistent and transient infection<br>- Proficiency panel of different Pestivirus strains available<br>- Detection of viral RNA in skin biopsy samples unaffected by maternally-derived antibodies, therefore allows for identification of persistently infected animals early in life<br>- Successfully applied in ongoing or completed control programmes | - Possibility for contamination at sample collection or in laboratory, leading to false positive results<br>- Needs specialised equipment<br>- Detection of viral RNA does not imply per se that infectious virus is present | - Presi & Heim (2010). *Vet. Microbiol.*, **142**, 137–142<br>- Schweizer *et al.* (2021) *Front. Vet. Sci.*, **8**, 702730<br>- Wernike *et al.* (2017). *Pathogens*, **6** (4)<br>- Graham *et al.* (2021) *Front. Vet. Sci.*, **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. | | | - Simple to perform and cost-effective<br><br>- Milk collection is non-invasive method with potential for herd screening with tank/bulk milk samples<br><br>- Bulk milk sensitive indicator for PI in herd | - Some cross-reactivity with vaccines and other pestiviruses<br><br>- PI animal will usually be seronegative<br><br>- Bulk milk from herd excludes males, non-lactating or young stock | Beaudeau *et al.* (2001). *Vet. Microbiol.*, **80**, 329–337<br>Lanyon *et al.* (2013). *Aust. Vet. J.*, **91**, 52–56. |

| 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 |
|---|---|---|---|---|---|---|---|
| Antigen detection by ELISA +++ | Serum, whole blood, skin biopsy | DSe 67–100% and DSp 98.8–100% relative to virus isolation reported | | | 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. | Maternal antibodies in colostrum may interfere with testing for antigen in serum using ELISA in calves. Ear notch samples are less affected. PI calves *in utero* defies detection. | Lanyon *et al.* (2013). *Vet.. J.* **199**, 201–209; |
| Virus isolation + | Serum, whole blood | Considered (historically) reference test; DSe <90% compared with real-time RT-PCR ; DSp ~100% | N/A | Historical information with no formal validation | - High degree of specificity<br>- Identifies presence of infectious virus | - Requires specialised cell culture capabilities and access to BVDV free materials<br>- Reduced sensitivity in presence of maternally-derived antibodies | N/A |
| Virus neutralisation test + | Serum | DSe & DSp both extremely high, both >99%. Historical reference serological test. | N/A | Historical information with no formal validation | Very high specificity | - ASe can vary depending on virus strain used<br>- Requires cell culture, good quality samples<br>- Labour intensive, takes 5 days to obtain results<br>- Expensive | N/A |

18 N/A: not available

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# CHAPTER 3.4.7.

# BOVINE VIRAL DIARRHOEA

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

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

# A. INTRODUCTION

## 1. Impact of the disease

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

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

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

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

## 2. The causal agent

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

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

## 3. Pathogenesis

### 3.1. Acute infections

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

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

### 3.2. *In-utero* infections

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

### 3.3. Persistent infections

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

### 3.4. Mucosal disease

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

### 3.5. Semen and embryos

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

Biological materials used for *in-vitro* fertilisation techniques (bovine serum, bovine cell cultures) have a high risk of contamination and should be screened for BVDV. Incidents of apparent introduction of virus via such techniques have highlighted this risk. It is considered essential that serum supplements used in media should be free of contaminants as detailed in Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use,* using techniques described in Section B.~~3~~ 1.1 of this chapter.

## 4. Approaches to diagnosis and sample collection

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

### 4.1. Acute infections

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

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

### 4.2. Persistent infections

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

### 4.3. Mucosal disease

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

### 4.4. Reproductive materials

Semen donor bulls should be sampled for testing for freedom from BVDV infection prior to collection of semen, in accordance with the *Terrestrial Animal Health Code.* It is necessary to confirm that these bulls

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

## B. DIAGNOSTIC TECHNIQUES

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

| Method | Purpose | | | | | |
|---|---|---|---|---|---|---|
| | Population freedom from infection[(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 | +++ | ++ | +++ | – +[(g)] | +++ | +++ |
| 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 method; NA = nucleic acid; RT-PCR = reverse-transcription polymerase chain reaction; ISH = *in-situ* hybridisation; VN = virus neutralisation.
[(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)]See Appendix 3 of this chapter for justification table for the scores giving to the tests for this purpose.
[(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 detection methods applied on the same clinical sample is recommended.

### 1. Detection of the agent

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

essential component of virus isolation in cell culture to detect the presence of non-cytopathic strains of BVDV which predominate in field infections.

All test methods must be extensively validated by testing on known uninfected and infected populations of cattle, including animals with low- and high-titre viraemias. Methods based on polyclonal or MAb-binding assays (ELISA or IHC), immune labelling (VI) or on nucleic acid recognition (PCR) must be shown to detect the full range of antigenic and genetic diversity found among BVD viruses. There are ~~three~~ designated WOAH 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.

### 1.1. Virus isolation

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

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

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

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

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

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

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

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

iii) The plate is incubated at 37°C for 4 days, either in a 5% $CO_2$ atmosphere or with the plate sealed.

iv) Each well is examined microscopically for evidence of cytopathology (cytopathic effect or CPE), or signs of cytotoxicity.

v) The cultures are frozen briefly at approximately –80°C and 50 µl of the culture supernatant is passaged to new cell cultures, repeating steps ~~3~~1.1.1.i to iv above.

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

- **Paraformaldehyde**

a) Add 200 µl of a 1/10 dilution of formaldehyde solution (approximately 3% concentration) to the plate and leave at room temperature for 10 minutes.

b) The contents of the plate are then discarded and the plate is washed.

c) Wash plates 5 times with 0.05% Tween 20 in water (an automatic microplate washer can be used with a low pressure and speed setting).

d) To each well add 50 µl of an antiviral antibody at the appropriate dilution (prepared in phosphate buffered saline/ PBS containing 1% gelatin) and incubate for 60–90 minutes at 37°C in a humidified chamber.

e) Wash plates five times as in step c).

f) Dilute the appropriate peroxidase conjugated antiserum to the optimum dilution in 1% gelatin/PBS (e.g. peroxidase conjugated rabbit anti-mouse immunoglobulin when the antiviral antibody is a mouse monoclonal). The optimum concentration should be determined for each batch of conjugate by “checkerboard” titration against reference positive and negative controls.

g) To each well of the microplate add 50 µl of the diluted peroxidase conjugate and incubate for 90 minutes at 37°C in a humidified chamber.

h) Wash plates five times as in step c).

i) “Develop” the plate by adding 3-amino-9-ethyl carbazole (AEC) substrate (100 µl/well) and allowing to react for 30 minutes at room temperature.

j) Add 100 µl of PBS to each well and add a lid to each plate.

k) Examine the wells by light microscopy, starting with the negative and positive control wells. There should be no or minimal staining apparent in the cells that were uninfected (negative control). The infected (positive control) cells should show a reddish- brown colour in the cytoplasm.

- **Acetone**

a) The plate is emptied by gentle inversion and rinsed in PBS.

b) The cells are fixed as follows: the plate is dipped into a bath of 20% acetone in PBS, emptied immediately and then transferred to a fresh bath of 20% acetone in PBS for 10 minutes. The plate is drained thoroughly and as much fluid as possible is removed by tapping and blotting. The plate is dried thoroughly for at least 3 hours at a temperature of 25–30°C (e.g. using radiant heat from a bench lamp). *Note:* the drying is part of the fixation process.

c) The fixed cells are rinsed by adding PBS to all wells.

d) The wells are drained and the antiviral BVD antibody (50 µl) is added to all wells at a predetermined dilution in PBS containing 1% Tween 80 (PBST) and 5% horse serum or 1% gelatin. (Horse serum or gelatin may be added to reduce nonspecific staining.)

e) Incubate at 37°C for 15 minutes.

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

g) Drain and add the appropriate anti-species serum conjugated to peroxidase at a predetermined dilution in PBST (50 µl per well) for 15 minutes at 37°C.

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

i) Rinse the plate in distilled water. Ensure all fluid is tapped out from the plate.

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

An alternative substrate can be made, consisting of 9 mg diaminobenzidine tetrahydrochloride and 6 mg sodium perborate tetrahydrate dissolved in 15 ml of PBS. Though the staining is not quite so intense, these chemicals have the advantage that they can be shipped by air.

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

Alternative methods for fixation of the cells may be used and include the use of heat (see Chapter 3.8.3 *Classical swine fever*, Section B.2.2.1.viii). These should be first evaluated to ensure that the capacity to detect viral antigen is not compromised.

#### 1.1.2. Tube method for tissue or buffy coat suspensions

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

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

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

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

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

v) The culture should then be frozen and thawed for passage to fresh cultures for one or preferably two more passages (including the culture inoculated for the final immunostaining). At the final passage, after freeze–thaw the tissue culture fluid is harvested and passaged on to microtitre plates for culture and staining by the immunoperoxidase method (see section B.~~3~~1.1.1 above) or by the immunofluorescent method. For immunofluorescence, cover-slips are included in the tubes and used to support cultured cells. At the end of the culture period, the cover slips are removed, fixed in 100% acetone and stained with an immunofluorescent conjugate to BVDV. Examine the cover slips under a fluorescent microscope for diffuse, cytoplasmic fluorescence characteristic of pestiviruses. Alternatively, culture supernatant from the final passage can be screened by real-time RT-PCR (see below).

#### 1.1.3. Virus isolation from semen

The samples used for the test are, typically, extended bovine semen or occasionally raw semen. Semen samples should be transported to the laboratory in liquid nitrogen, or on dry ice. The

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

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

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

iii) Inoculate 1 ml of the semen/serum mixture into a monolayer of susceptible cells (see virus isolation from tissue above) in cell culture tubes or a six-well tissue culture plate.

iv) Incubate the cultures for 1 hour at 37°C.

v) Remove the mixture, wash the monolayer several times with maintenance medium and then add new maintenance medium to the cultures.

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

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

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

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

### 1.2. Nucleic acid detection

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

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

A variety of commercially available nucleic acid purification systems are available in kit form, and several can be semi-automated. Systems based on the capture and purification of RNA using magnetic beads are in widespread use and allow rapid processing of large numbers of samples. Specific products should be evaluated to determine the optimal kit for a particular sample type and whether any preliminary sample processing is required. For whole blood samples, the type of anticoagulant and volume of blood in a specimen tube is important. More problems with inhibitors of the PCR reaction are encountered with samples collected into heparin treated blood than EDTA. These differences are also exacerbated if the tube does not contain the recommended volume of blood, thereby increasing the concentration of anticoagulant in the sample. To identify possible false-negative results, it is recommended to spike an exogenous ('internal control') RNA template into the specimen prior to RNA extraction (e.g. Hoffman *et al*., 2006). By the inclusion of PCR primers and probe specific to the exogenous sequence, the efficiency of both the RNA extraction and also the presence of any PCR inhibitors can be monitored. While valuable for all sample types, the inclusion of an internal control is particularly desirable when testing semen and whole blood. When using an internal control, extensive testing is necessary to ensure that PCR amplification of the internal control does not compete with the diagnostic PCR and thus lower the analytical sensitivity (see also chapter 1.1.6).

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

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

Real-time RT-PCR has been shown to be extremely useful to screen semen samples to demonstrate freedom from BVDV and, apart from speed, often gives superior results to virus isolation in cell culture, especially when low virus levels are present, such as may be found in bulls with a PTI. The real-time RT-PCR described here uses a pair of sequence-specific primers for amplification of target ~~D~~ RNA and a 5'-nuclease oligoprobe for the detection of amplified products. The oligoprobe is a single, sequence-specific oligonucleotide, labelled with two different fluorophores. The primers and probe are available commercially and several different fluorophores options are available. This pan-pestivirus real-time RT-PCR assay is designed to detect viral ~~D~~ RNA of all strains of BVDV types 1 (*Pestivirus bovis*)~~ and BVDV~~, 2 (*Pestivirus tauri*) and 3 (*Pestivirus brazilense*) as well as ~~BDV,~~ CSFV (*Pestivirus suis*), some strains of BDV (*Pestivirus ovis*) and most atypical pestiviruses. The assay selectively amplifies a 208 base pair sequence of the 5' non-translated region (5' NTR) of the pestivirus genome. Details of the primers and probes are given in the protocol outlined below.

i) Sample preparation, equipment and reagents

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

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

c) A real-time PCR detection system, and the associated data analysis software, is required to perform the assay. A number of real-time PCR detection systems are available from various manufacturers. ~~Other equipment required for the test includes a micro-centrifuge, a chilling block, a micro-vortex, and micropipettes.~~ As real-time RT-PCR assays are able to detect very small amounts of target nucleic acid molecules, appropriate measures are required to avoid contamination. ~~, including dedicated and physically separated 'clean' areas for reagent preparation (where no samples or materials used for PCR are handled), a dedicated sample processing area and an isolated area for the PCR thermocycler and associated equipment. Each area should have dedicated reagents and equipment.~~ Furthermore, a minimum of one negative sample should be processed in parallel to monitor the possibility of low level contamination. Sources of contamination may include product carry-over from positive samples or, more commonly, from cross contamination by PCR products from earlier work.

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

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

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

ii) Extraction of RNA

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

iii) Real-time RT-PCR assay procedure

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

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

c) Primers and probe sequences

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

*Forward:* BVD 190-F 5'-GRA-GTC-GTC-ART-GGT-TCG-AC

*Reverse:* V326 5'-TCA-ACT-CCA-TGT-GCC-ATG-TAC

*Probe:* TQ-pesti 5'-FAM-TGC-YAY-GTG-GAC-GAG-GGC-ATG-C-TAMRA-3'

d) Preparation of reaction mixtures

The PCR reaction mixtures are prepared in a separate room that is isolated from other PCR activities and sample handling. For each PCR test, appropriate controls should be included. As a minimum, a no template control (NTC), appropriate negative control (NC) and two positive controls (PC1, PC2) should be included. The positive and negative controls are included in all steps of the assay from extraction onwards while the NTC is added after completion of the extraction. The PCR amplifications are carried out in a volume of 25 µl.

The protocol described is based on use of a 96 well microplate based system but other options using microtubes are also suitable. Each well of the PCR plate should contain 20 µl of reaction mix and 5 µl of sample as follows:

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

e) Selection of controls

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

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

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

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

g) Real-time polymerase chain reaction

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

1× 48°C 10 minutes

1× 95°C 10 minutes

45 × (95°C 15 seconds, 60°C 1 minute)

h) Analysis of real-time PCR data

The software program is usually set to automatically adjust results by compensating for any background signal and the threshold level is usually set according to the manufacturer's instructions for the selected analysis software used. In this instance, a threshold is set at 0.05.

i) Interpretation of results

a) Test controls – all controls should give the expected results with positive controls (PC1 and PC2) falling within the designated range and both the negative control (NC) and no template control (NTC) should have no Ct values.

b) Test samples

1) Positive result: Any sample that has a cycle threshold (Ct) value less than 40 is regarded as positive.

2) Negative result: Any sample that shows no Ct value is regarded as negative. However, before reporting a negative result for a sample, the performance of the exogenous internal control should be checked and shown to give a result within the accepted range for that control (for example, a Ct value no more than 2–3 Ct units higher than the NTC).

### 1.3. Enzyme-linked immunosorbent assay for antigen detection

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

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

### 1.4. Immunohistochemistry

Enzyme-labelled methods are useful to detect BVDV antigen in tissue sections, particularly where suitable MAbs are available. However, these assays are not appropriate to certify animals for international trade and use should be limited to diagnostic investigations. It is important that the reagents and procedures used be fully validated, and that nonspecific reactivity be eliminated. For PI cattle almost any tissue can be used, but particularly good success has been found with lymph nodes, thyroid gland, skin, brain, abomasum and placenta. Skin biopsies, such as ear-notch samples, have shown to be useful for *in-vivo* diagnosis of persistent BVDV infection.

## 2. Serological tests

Antibody to BVDV can be detected in cattle sera by a standard VNT or by ELISA, using one of several published methods or with commercial kits (e.g. Edwards, 1990). Serology is used to identify levels of herd immunity, for the detection of the presence of PI animals in a herd, to assist with investigation of reproductive disease and possible involvement of BVDV and to establish the serological status of bulls being used for semen collection and to identify whether there has been a recent infection. ELISA for antibody in bulk milk samples can give a useful indication of the BVD status of a herd (Niskanen, 1993). High ELISA values ~~(0.8 or more absorbance units)~~ in an unvaccinated herd indicates a high probability of the herd having been exposed to BVDV in the recent past, most likely through one or more persistently viraemic animals being present. In contrast, ~~a~~ very low or negative values ~~(≤0.2)~~ indicate~~s~~ that it is unlikely that persistently viraemic animals are present. However, ELISA values are not always a reliable indicator of the presence of PI animals on farms, due to differing husbandry (Zimmer *et al*., 2002), recent administration of vaccine and also due to the presence of viral antigen in bulk milk, which may interfere with the antibody assay itself. Determination of the antibody status of a small number of young stock (9–18 months) has also been utilised as an indicator of recent transmission of BVDV in the herd (Houe *et al*., 1995), but this approach

738 is also dependent on the degree of contact between different groups of animals in the herd and the potential for
739 exposure from neighbouring herds. VN tests are more frequently used for regulatory purposes (e.g. testing of semen
740 donors) while ELISAs (usually in the form of commercially prepared kits) are commonly used for diagnostic
741 applications. Whether ELISA or VNT, control positive and negative standard sera must be included in every test.
742 These should give results within predetermined limits for the test to be considered valid. In the VNT, a 'serum
743 control' to monitor sample toxicity should also be included for each test sample.

744 ## 2.1. Virus neutralisation test

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

757 ### 2.1.1. Test procedure

758 i) The test sera are heat-inactivated for 30 minutes at 56°C.

759 ii) From a starting dilution of 1/4, serial twofold dilutions of the test sera are made in a cell-
760 culture grade flat-bottomed 96-well microtitre plate, using cell culture medium as diluent. For
761 each sample, three or four wells are used at each dilution depending on the degree of
762 precision required. At each dilution of serum, for each sample one well is left without virus
763 to monitor for evidence of sample toxicity that could mimic viral cytopathology or interfere
764 with virus replication. Control positive and negative sera should also be included in each
765 batch of tests.

766 iii) An equal volume (e.g. 50 µl) of a stock of cytopathic strain of BVDV containing 100 $TCID_{50}$
767 (50% tissue culture infective dose) is added to each well. A back titration of virus stock is
768 also done in some spare wells to check the potency of the virus (acceptance limits 30–
769 300 $TCID_{50}$).

770 iv) The plate is incubated for 1 hour at 37°C.

771 v) A flask of suitable cells (e.g. bovine turbinate, bovine testis) is trypsinised and the cell
772 concentration is adjusted to 1.5 × $10^5$/ml. 100 µl of the cell suspension is added to each well
773 of the microtitre plate.

774 vi) The plate is incubated at 37°C for 4–5 days, either in a 5% $CO_2$ atmosphere or with the plate
775 sealed.

776 vii) The wells are examined microscopically for CPE or fixed and stained by immunoperoxidase
777 staining using an appropriate monoclonal antibody. The VN titre for each serum is the
778 dilution at which the virus is neutralised in 50% of the wells. This can be calculated by the
779 Spearman–Kärber or Reed Muench methods. A seronegative animal will show no
780 neutralisation at the lowest dilution (1/4), equivalent to a final dilution of 1/8. For accurate
781 comparison of antibody titres, and particularly to demonstrate significant (more than fourfold)
782 changes in titre, samples should be tested in parallel in the same test.

783 ## 2.2. Enzyme-linked immunosorbent assay

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

789 The chief difficulty in setting up the test lies in the preparation of a viral antigen of sufficient potency. The
790 virus must be grown under optimal culture conditions using a highly permissive cell type. Any serum
791 used in the medium must not inhibit growth of BVDV. The optimal time for harvest should be determined
792 experimentally for the individual culture system. The virus can be concentrated and purified by density
793 gradient centrifugation. Alternatively, a potent antigen can be prepared by treatment of infected cell

cultures with detergents, such as Nonidet P40, N-decanoyl-N-methylglucamine (Mega 10), Triton X-100 or 1-octylbeta-D-glucopyranoside (OGP). Some workers have used fixed, infected whole cells as antigen. ~~In the future,~~ Increasing use ~~may be~~ is made of artificial antigens manufactured by expressing specific viral genes in bacterial or eukaryotic systems. Such systems should be validated by testing sera specific to a wide range of different virus strains. In the future, this technology should enable the production of serological tests complementary to subunit or marker vaccines, thus enabling differentiation between vaccinated and naturally infected cattle. An example outline protocol for an indirect ELISA is given below (Edwards, 1990).

#### 2.2.1. Test procedure

i) Roller cultures of secondary calf testis cells with a high multiplicity of infection (about one), are inoculated with BVDV strain Oregon C24V, overlaid with serum-free medium and incubated for 24 hours at 37°C.

ii) The cells are scraped off and pelleted. The supernatant medium is discarded. The pellet is treated with two volumes of 2% OGP in PBS for 15 minutes at 4°C, and centrifuged to remove the cell debris. The supernatant antigen is stored in small aliquots at –70°C, or freeze-dried. Non-infected cells are processed in parallel to make a control antigen.

iii) The antigen is diluted to a predetermined dilution in 0.05 M bicarbonate buffer, pH 9.6. Alternate rows of an ELISA-grade microtitre plate are coated with virus and control antigens overnight at 4°C. The plates are then washed in PBS with 0.05% Tween 20 or Tween 80 (PBST) before use in the test.

iv) Test sera are diluted 1/50 in serum diluent (0.5 M NaCl; 0.01 M phosphate buffer; 0.05% Tween 20; 0.001 M ethylene diamine tetra-acetic acid; 1% polyvinyl pyrrolidone, pH 7.2) and added to virus- and control-coated wells for 1 hour at 37°C. The plates are then washed five times in PBST.

v) Rabbit anti-bovine IgG peroxidase conjugate is added at a predetermined dilution (in serum diluent) for 1 hour at 37°C, then the plates are again washed five times in PBST.

vi) A suitable enzyme substrate is added, such as hydrogen peroxide/tetramethyl benzidine. After colour development, the reaction is stopped with sulphuric acid and the absorbance is read on an ELISA plate reader. The value obtained with control antigen is subtracted from the test reaction to give a net absorbance value for each serum.

vii) It is recommended to convert net absorbance values to sample:positive ratio (or percentage positivity) by dividing net absorbance by the net absorbance on that test of a standard positive serum that has a net absorbance of about 1.0. This normalisation procedure leads to more consistent and reproducible results.

# C. REQUIREMENTS FOR VACCINES

## 1. Background

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

### 1.1. Characteristics of a target product profile

Traditionally, BVD vaccines fall into two classes: modified live or inactivated virus vaccines. The essential requirement for both types is to ~~afford~~ provide a high level of fetal ~~infection~~ protection. Many of the live vaccines have been based on a cytopathic strain of the virus which is considered to be unable to cross

the placenta. However, it is important to ensure that the vaccine virus does not cause fetal infection. In general, vaccination of breeding animals should be completed well before insemination to ensure optimal protection and avoid any risk of fetal infection. Live virus vaccine may also be immunosuppressive and precipitate other infections. On the other hand, modified live virus vaccines may only require a single dose. Use of a live product containing a cytopathic strain of BVDV may precipitate mucosal disease by superinfection of persistently viraemic animals. Properly formulated inactivated vaccines are very safe to use but, to obtain satisfactory levels of immunity, they usually require booster vaccinations, which may be inconvenient. A combined vaccination protocol using inactivated followed by live vaccine may reduce the risk of adverse reaction to the live strain. Whether live or inactivated, because of the propensity for antigenic variability, the vaccine should contain strains of BVDV that are closely matched to viruses found in the area in which they are used. For example, in countries where strains of BVDV type 2 (*Pestivirus tauri*) are found, it is important for the vaccine to contain a suitable type 2 strain. For optimal immunity against type 1 strains (*Pestivirus bovis*), antigens from the dominant subtypes (e.g. 1a and 1b) should be included. Due to the need to customise vaccines for the most commonly encountered strains within a country or region, it is not feasible to produce a vaccine antigen bank that can be drawn upon globally.

Guidance for the production of veterinary vaccines is given in Chapter 1.1.8 *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.

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

### 2.1. Characteristics of the seed

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

#### 2.1.1. Biological characteristics of the master seed

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

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

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

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

904 **2.1.3. Validation as a vaccine strain**

905–913 All vaccines should pass standard tests for efficacy. Tests should include as a minimum the demonstration of a neutralising antibody response following vaccination, a reduction in virus shedding after challenge in vaccinated cattle and ideally a prevention of viraemia. Efficacy tests of BVD vaccines by assessing clinical parameters in non-pregnant cattle can be limited by the difficulty of consistently establishing clinical signs but, when employed, clinical parameters such as a reduction in the rectal temperature response and leukopenia should be monitored. Although it can be difficult by using virus isolation in cell culture to consistently demonstrate the low levels of viraemia associated with an acute infection, real-time PCR could be considered as an alternative method to establish the levels of circulating virus.

914–922 If a vaccine passes basic tests, the efficacy of vaccination should ultimately be measured by the capacity to prevent transplacental transmission. If there is a substantial reduction and ideally complete prevention of fetal infection, a vaccine would be expected to be highly effective in other situations (for example prevention of respiratory disease). A suitable challenge system can be established by intranasal inoculation of noncytopathic virus into pregnant cows between 60 and 90 days of gestation (Brownlie *et al*., 1995). Usually this system will reliably produce persistently viraemic offspring in non-immune cows. In countries where BVDV type 2 viruses (*Pestivirus tauri*) are commonly encountered, efficacy in protecting against BVDV type 2 infections should be measured.

923 **2.2. Method of manufacture**

924 **2.2.1. Procedure**

925–932 Both cytopathic and noncytopathic biotypes will grow in a variety of cell cultures of bovine origin. Standard procedures may be used, with the expectation for harvesting noncytopathic virus on days 4–7 and cytopathic virus on days 2–4. The optimal yield of infectious virus will depend on several factors, including the cell culture, isolate used and the initial seeding rate of virus. These factors should be taken into consideration and virus replication kinetics investigated to establish the optimal conditions for large scale virus production. Whether a live or inactivated vaccine, the essential aim will be to produce a high-titred virus stock. This bulk antigen preparation can subsequently be prepared according to the type of vaccine being considered.

933 **2.2.2. Requirements for ingredients**

934–942 Most BVDV vaccines are grown in cell cultures of bovine origin that are frequently supplemented with medium components of animal origin. The material of greatest concern is bovine serum due to the potential for contamination with BVD viruses and antibodies to these viruses. These adventitious contaminants not only affect the efficiency of production but also may mask the presence of low levels of infectious BVDV that may have undesirable characteristics. In addition to the virus seeds, all materials should be tested for sterility and freedom from contamination with other agents, especially viruses as described in chapters 1.1.8 and 1.1.9. Further, materials of bovine or ovine origin should originate from a country with negligible risk for transmissible spongiform encephalopathies [TSEs] (see chapter 1.1.9).

943 **2.2.3. In-process controls**

944–958 In-process controls are part of the manufacturing process. Cultures should be inspected regularly to ensure that they remain free from contamination, and to monitor the health of the cells and the development or absence of CPE, as appropriate. While the basic requirement for efficacy is the capacity to induce an acceptable neutralising antibody response, during production, target concentrations of antigen required to achieve an acceptable response may be monitored indirectly by assessment of the quantity of infectious virus or antigen mass that is produced. Rapid diagnostic assays such as the ELISA are useful to monitor BVDV antigen production. Alternatively, the quality of a batch of antigen may be determined by titration of the quantity of infectious virus present, although this may underestimate the quantity of antigen. For inactivated vaccines, infectivity is evaluated before inactivation. For inactivated vaccines the inactivation kinetics should be established so that a suitable safety margin can be determined and incorporated into the routine production processes. At the end of production, *in-vitro* cell culture assays should be undertaken to confirm that inactivation has been complete. These innocuity tests should include a sufficient number of passages and volume of inoculum to ensure that very low levels of infectious virus would be detected if present.

#### 2.2.4. Final product batch tests

i) Sterility

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in Chapter 1.1.9.

ii) Identity

Identity tests should demonstrate that no other strain of BVDV is present when several strains are propagated in a facility producing multivalent vaccines.

iii) Safety

Safety tests shall consist of detecting any abnormal local or systemic adverse reactions to the vaccine by all vaccination route(s). Batch-to-batch safety tests are required unless safety of the product is demonstrated and APPROVED in the registration dossier and production is consistent with that described in chapter 1.1.8.

The safety test is different to the inocuity test (see above).

Live vaccines must either be demonstrated to be safe in pregnant cattle (i.e. no transmission to the fetus), or should be licensed with a warning not to use them in pregnant animals. Live vaccines containing cytopathic strains should have an appropriate warning of the risk of inducing mucosal disease in PI cattle.

iv) Batch potency

BVD vaccines must be demonstrated to produce adequate immune responses, when used in their final formulation according to the manufacturer's published instructions. The minimum quantity of infectious virus and/or antigen required to produce an acceptable immune response should be determined. *In-vitro* assays should be used to monitor individual batches during production.

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

#### 2.3.1. Manufacturing process

For registration of a vaccine, all relevant details concerning manufacture of the vaccine and quality control testing should be submitted to the relevant authorities. Unless otherwise specified by the authorities, information should be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

There is no standard method for the manufacture of a BVD vaccine, but conventional laboratory techniques with stationary, rolled or suspension (micro-carriers) cell cultures may be used. Inactivated vaccines can be prepared by conventional methods, such as binary ethylenimine or beta-propiolactone inactivation (Park & Bolin, 1987). A variety of adjuvants may be used.

#### 2.3.2. Safety requirements

*In-vivo* tests should be undertaken using a single dose, overdose (for live vaccines only) and repeat doses (taking into account the maximum number of doses for primary vaccination and, if appropriate, the first revaccination/booster vaccination) and contain the maximum permitted antigen load and, depending on the formulation of the vaccine, the maximum number of vaccine strains.

i) Target and non-target animal safety

The safety of the final product formulation of both live and inactivated vaccines should be assessed in susceptible young calves that are free of maternally derived antibodies and in pregnant cattle. They should be checked for any local reactions following administration, and, in pregnant cattle, for any effects on the unborn calf. Live attenuated vaccines may contribute to immunosuppression that might increase mortality. It may also contribute to the development of mucosal disease in PI animals that is an animal welfare concern. Therefore vaccination of PI animals with live attenuated vaccines containing cytopathic BVDV should be avoided. Live attenuated vaccines must not be capable of being transmitted to other unvaccinated animals that are in close contact.

1008 ii) Reversion-to-virulence for attenuated/live vaccines and environmental considerations

1009–1013 Virus seeds that have been passaged at least up to and preferably beyond the passage limit specified for the seed should be inoculated into young calves to confirm that there is no evidence of disease. If a live attenuated vaccine has been registered for use in pregnant animals, reversion to virulence tests should also include pregnant animals. Live attenuated vaccines should not be transmissible to unvaccinated 'in-contact' animals.

1014 iii) Precautions (hazards)

1015–1021 BVDV is not considered to be a human health hazard. Standard good microbiological practice should be adequate for handling the virus in the laboratory. A live virus vaccine should be identified as harmless for people administering the product. However adjuvants included in either live or inactivated vaccines may cause injury to people. Manufacturers should provide adequate warnings that medical advice should be sought in the case of self-injection (including for adjuvants, oil-emulsion vaccine, preservatives, etc.) with warnings included on the product label/leaflet so that the vaccinator is aware of any danger.

#### 1022 2.3.3. Efficacy requirements

1023–1030 The potency of the vaccine should be determined by inoculation into seronegative and virus negative calves, followed by monitoring of the antibody response. Antigen content can be assayed by ELISA and adjusted as required to a standard level for the particular vaccine. Standardised assay protocols applicable to all vaccines do not exist. Live vaccine batches may be assayed by infectivity titration. Each production batch of vaccine should undergo potency and safety testing as batch release criteria. BVD vaccines must be demonstrated to produce adequate immune responses, as outlined above, when used in their final formulation according to the manufacturer's published instructions.

#### 1031 2.3.4. Vaccines permitting a DIVA strategy (detection of infection in vaccinated animals)

1032–1037 To date, there are no commercially available vaccines for BVDV that support use of a true DIVA strategy. Experimental subunit vaccines based on baculovirus-expressed BVD viral glycoprotein E2 have been described but are not available commercially. They offer a future prospect of 'marker vaccines' when used in connection with a complementary serological test. Experimental BVDV E2 DNA vaccines and BVDV E2 subunit vaccines expressed using transgenic plants and alphavirus replicon or chimeric pestivirus vaccines have also been described.

#### 1038 2.3.5. Duration of immunity

1039–1047 There are few published data on the duration of antibody following vaccination with a commercial product. Protocols for their use usually recommend a primary course of two inoculations and boosters at yearly intervals. Only limited data are available on the antibody levels that correlate with protection against respiratory infections (Bolin & Ridpath, 1995; Howard *et al.*, 1989) or *in-utero* infection (Brownlie *et al.*, 1995). However, there are many different commercial formulations and these involve a range of adjuvants that may support different periods of efficacy. Consequently, duration of immunity data must be generated separately for each commercially available product by undertaking challenge tests at the end of the period for which immunity has been claimed.

#### 1048 2.3.6. Stability

1049–1055 There are no accepted guidelines for the stability of BVD vaccines, but it can be assumed that attenuated virus vaccine (freeze-dried) should remain potent for at least 1 year if kept at 4°C. Inactivated virus vaccine could have a longer shelf life at 4°C. Lower temperatures could prolong shelf life for either type, but adjuvants in killed vaccine may preclude this. Bulk antigens that have not been formulated into finished vaccine can be reliably stored frozen at low temperatures but the antigen quality should be monitored with *in-vitro* assays prior to incorporation into a batch of vaccine.

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1142 **NB:** There are WOAH Reference Laboratories for bovine viral diarrhoea (please consult the WOAH Web site:
1143 https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3 )
1144 Please contact the WOAH Reference Laboratories for any further information on
1145 diagnostic tests, reagents and vaccines for bovine viral diarrhoea

1146 **NB:** FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2015.

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## 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 | - Very sensitive<br>- Rapid<br>- High-throughput<br>- Well established internationally<br>- Detects assay-dependent all BVDV species<br>- Allows assay-dependent for differentiation of BVDV types 1 and 2<br>- Detects persistent and transient infection<br>- Proficiency panel of different Pestivirus strains available<br>- Detection of viral RNA in skin biopsy samples unaffected by maternally-derived antibodies, therefore allows for identification of persistently infected animals early in life<br>- Successfully applied in ongoing or completed control programmes | - Possibility for contamination at sample collection or in laboratory, leading to false positive results<br>- Needs specialised equipment<br>- Detection of viral RNA does not imply per se that infectious virus is present | - Presi & Heim (2010). *Vet. Microbiol.*, **142**, 137–142<br>- Schweizer *et al.* (2021) *Front. Vet. Sci.*, **8**, 702730<br>- Wernike *et al.* (2017). *Pathogens*, **6** (4)<br>- Graham *et al.* (2021) *Front. Vet. Sci.*, **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. | | | - Simple to perform and cost-effective<br><br>- Milk collection is non-invasive method with potential for herd screening with tank/bulk milk samples<br><br>- Bulk milk sensitive indicator for PI in herd | - Some cross-reactivity with vaccines and other pestiviruses<br><br>- PI animal will usually be seronegative<br><br>- Bulk milk from herd excludes males, non-lactating or young stock | Beaudeau *et al.* (2001). *Vet. Microbiol.*, **80**, 329–337<br><br>Lanyon *et al.* (2013). *Aust. Vet. J.*, **91**, 52–56. |

| **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** |
|---|---|---|---|---|---|---|---|
| Antigen detection by ELISA +++ | Serum, whole blood, skin biopsy | DSe 67–100% and DSp 98.8–100% relative to virus isolation reported | | | 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. | Maternal antibodies in colostrum may interfere with testing for antigen in serum using ELISA in calves. Ear notch samples are less affected. PI calves *in utero* defies detection. | Lanyon *et al.* (2013). *Vet.. J.* **199**, 201–209; |
| Virus isolation + | Serum, whole blood | Considered (historically) reference test; DSe <90% compared with real-time RT-PCR ; DSp ~100% | N/A | Historical information with no formal validation | - High degree of specificity<br>- Identifies presence of infectious virus | - Requires specialised cell culture capabilities and access to BVDV free materials<br>- Reduced sensitivity in presence of maternally-derived antibodies | N/A |
| Virus neutralisation test + | Serum | DSe & DSp both extremely high, both >99%. Historical reference serological test. | N/A | Historical information with no formal validation | Very high specificity | - ASe can vary depending on virus strain used<br>- Requires cell culture, good quality samples<br>- Labour intensive, takes 5 days to obtain results<br>- Expensive | N/A |

3 N/A: not available

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5

# Appendix 2: Bovine viral diarrhoea

## Intended purpose of test: individual animal freedom from infection prior to movement

| 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 |
|---|---|---|---|---|---|---|---|
| Virus isolation ++ | Serum, whole blood. | Considered reference test; DSe <90% compared with real-time RT-PCR; DSp ~100% | N/A | Historical information with no formal validation | - High degree of specificity<br>- Identifies presence of infectious virus | - Requires specialised cell culture capabilities and access to BVDV free materials<br>- Reduced sensitivity in presence of MDA (diagnostic gap); takes several weeks for maximum DSe | Edmonson *et al.* (2007); Toker & Yesilbag (2021) |
| Antigen detection by ELISA +++ | Serum, whole blood, skin biopsy (e.g. ear notch) | DSe 67–100% and DSp 98.8–100% relative to virus isolation have been reported but usually DSe and DSp are extremely high | | | Relatively simple to perform, rapid, can be 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 *in utero* defies detection. | Zimmer *et al.* (2004). *Vet. Microbiol.*, **100**, 145–149 |
| NA detection by (real-time) RT-PCR +++ | Ear notch (skin), blood; nasal or oral swab | - Depending on the assay analytical sensitivity of less than 10 genome copies/reaction<br>- Accuracy 98.73% based on a proficiency test panel consisting of five ear notch and five serum samples | | See references | - Very sensitive<br>- Rapid<br>- High-throughput<br>- Well established internationally<br>- Depending on the assay detects all BVDV species<br>- Allows assay-dependent for differentiation of BVDV types 1 and 2<br>- Detects persistent and transient infection<br>- Proficiency panel of different Pestivirus strains available<br>- Detection of viral RNA in skin biopsy samples unaffected by maternally-derived antibodies | - Possibility for contamination at sample collection or in laboratory, leading to false positive results<br>- Needs specialised equipment | - Hoffmann *et al.* (2006). *J. Virol. Methods*, **136**, 200–209.<br>- Wernike *et al.* (2019). *Vet. Microbiol.*, **239**, 108452. |

| 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 |
|---|---|---|---|---|---|---|---|
| Virus neutralisation test ++ | Serum | DSe & DSp both extremely high, both >99%. Historical reference serological test. | N/A | Historical information with no formal validation | Very high specificity<br>- Negative results generally indicate that an animal has not been infected provided it has been tested for virus/antigen<br>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. | - ASe can vary depending on virus strain used<br>- Requires cell culture, good quality samples<br>- time consuming to perform, takes 5 days to obtain results<br>- Labour intensive<br>- 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) | N/A |
| Antibody detection by ELISA ++ | Blood, Individual milk sample | 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. | | | - Simple to perform and cost-effective<br>- Milk collection is non-invasive method<br>- Paired samples can be used to confirm acute infection.<br>- Ensure milk is collected directly from the teat rather than bulk-milk tank to ensure no cross contamination/false-positives | - 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.<br>- PI animal will be seronegative and may impact receiving herds if moved.<br>- Using milk, limited to lactating cow only | N/A |

6 N/A: not available

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## Appendix 3: Bovine viral diarrhoea
## Intended purpose of test: contribute to eradication policies

| **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** |
|---|---|---|---|---|---|---|---|
| Antigen detection by ELISA +++ | Serum, whole blood, skin biopsy | DSse 67%–100% and DSp 98.8–100% reported | | | Relatively simple to perform, rapid, can be 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 *in utero* defy detection. | Zimmer *et al.* (2004). *Vet. Microbiol.*, **100**, 145–149 |
| NA detection by (real-time) RT-PCR +++ | Ear notch (skin), blood; milk; nasal or oral swab | Utility has been demonstrated under field conditions in large control programs | Whole Swiss, German and Irish cattle populations | See references | - Very sensitive<br>- Rapid<br>- High-throughput<br>- Well established internationally<br>- Depending on assay, detects all BVDV species<br>- Allows assay-dependent differentiation of BVDV types 1 and 2<br>- Detects persistent and transient infection<br>- Proficiency panel of different Pestivirus strains available<br>- Detection of viral RNA in skin biopsy samples unaffected from maternally-derived antibodies, therefore allows for identification of persistently infected animals early in life<br>- Successfully applied in ongoing or completed control programmes (see references) | - Possibility for contamination at sample collection or in laboratory, leading to false positive results<br>- Needs specialised equipment | - Presi & Heim (2010). *Vet. Microbiol.*, **142**, 137–142<br>- Schweizer *et al.* (2021). *Front. Vet. Sci.*, **8**, 702730<br>- Wernike *et al.* (2017). *Pathogens*, **6** (4)<br>- Graham *et al.* (2021). *Front. Vet. Sci.*, **8**, 674557 |

| 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 |
|---|---|---|---|---|---|---|---|
| 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. | | | - Simple to perform and cost-effective<br>- Milk collection is non-invasive method<br>- 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 | - Some cross-reactivity with antibodies from vaccines and other pestiviruses<br>- PI animal will be seronegative<br>- Bulk milk from herd does not include males, non-lactating or young stock | Laureyns *et al.* (2010) |
| Virus isolation ++ | Serum, whole blood | Considered reference test ; DSe <90% compared with real-time RT-PCR; DSp ~100% | N/A | Historical information with no formal validation | - High degree of specificity<br>- Identifies presence of infectious virus.<br>- used to confirm the status of difficult cases and to provide isolates for intensive analysis e.g. NA sequencing | - Requires specialised cell culture capabilities and access to BVDV free materials<br>- Reduced sensitivity in presence of MDA (diagnostic gap) | N/A |
| Virus neutralisation test ++ | Serum | DSe & DSp both extremely high, both >99%. Historical reference serological test. | N/A | Historical information with no formal validation | Very high specificity<br>- Used for confirming the virus free status of a population after eradication;<br>- Used as a confirmatory test when surveillance utilises an ELISA | - ASe can vary depending on virus strain used<br>- Requires cell culture, good quality samples<br>- Takes 5 days to obtain results | N/A |

10 N/A: not available

11 12

# Appendix 4: Bovine viral diarrhoea
## Intended purpose of test: confirmation of clinical cases

| 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 |
|---|---|---|---|---|---|---|---|
| Virus isolation ++ | Serum, whole blood, tissue extracts | Considered reference test; DSe <90% compared with real-time RT-PCR; DSp ~100% | Not available | Historical information with no formal validation | - High degree of specificity<br>- Identifies presence of infectious virus<br>- Preferred method to identify presence of cytopathogenic strains and hence confirmation of mucosal disease<br>- Provides virus isolates for detailed characterisation | - Requires specialised cell culture capabilities and access to BVDV free materials<br>- Reduced sensitivity in presence of maternally derived antibodies (diagnostic gap)<br>-Requires high quality samples to avoid bacterial contamination | – Meyling (1984) |
| Antigen detection by ELISA +++ | Serum, whole blood, skin biopsy | DSe 67%–100% and DSp 98.8% to 100% reported | | | Relatively simple to perform, rapid, can be 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 *in utero* defies detection. | |
| NA detection by (real-time) RT-PCR +++ | Blood; nasal, oral or conjunctival swab (in cases of respiratory disease) or faecal swab (enteric disease) | Depending on the assay analytical sensitivity of less than 10 genome copies/reaction | | See reference | - Very sensitive<br>- Rapid<br>- High-throughput<br>- Well established internationally<br>- Depending on the assay, detects all BVDV species<br>- Allows assay-dependent for differentiation of BVDV types 1 and 2<br>- Detects persistent and transient infection<br>- Proficiency panel of different Pestivirus strains available<br>- Detection of viral RNA in skin biopsy samples unaffected from maternally derived antibodies | - Possibility for contamination at sample collection or in laboratory, leading to false positive results<br>- Needs specialised equipment | - Hoffmann *et al.* (2006). *J. Virol. Methods*, **136**, 200–209. |
| Antigen detection by IHC ++ | Fixed tissues or frozen sections for Ag detection or NA if using ISH | Lower DSe than other methods; high DSp | N/A | N/A | Allows visualisation of viral components in lesions and assessment of tissue distribution | Technically demanding, limited reagents for detection of Ag in formalin-fixed tissues | |

| 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 |
|---|---|---|---|---|---|---|---|
| Antibody detection by ELISA + | Paired serum samples, fetal fluids (blood, pericardial, thoracic | 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. | | | - Simple to perform and cost-effective.<br>- Can be used to differentiate between acute and persistent infections by demonstration of seroconversion in acute infections<br>- Detection of antibodies in aborted fetuses, stillborn animals can confirm *in utero* infection in second half of gestation | - Some cross-reactivity with antibodies induced by other pestiviruses.<br>- PI animals are usually seronegative (in both of the paired samples) | |

13 N/A: not available

14
15

## Appendix 5: Bovine viral diarrhoea
## Intended purpose of test: prevalence of infection – surveillance

| 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 |
|---|---|---|---|---|---|---|---|
| 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 *in utero* defies detection. | Sarrazin *et al.* (2013). *Prev. Vet. Med.*, **108**, 28–37 |
| NA detection by (real-time) RT-PCR +++ | Ear notch (skin), blood, milk | | Whole Swiss, German and Irish cattle populations | See references | - Very sensitive<br>- Rapid<br>- High-throughput<br>- Well established internationally<br>- Depending on the assay, detects all BVDV species<br>- Allows assay-dependent for differentiation of BVDV types 1 and 2<br>- Detects persistent and transient infection<br>- Proficiency panel of different Pestivirus strains available<br>- Detection of viral RNA in skin biopsy samples unaffected from maternally-derived antibodies | - Possibility for contamination at sample collection or in laboratory, leading to false positive results<br>- Needs specialised equipment | - Presi & Heim (2010). *Vet. Microbiol.*, **142**, 137–142<br>- Schweizer *et al.* (2021). *Front. Vet. Sci.*, **8**, 702730<br>- Wernike *et al.* (2017) *Pathogens*, **6** (4)<br>- Graham *et al.* (2021). *Front. Vet. Sci.*, **8**, 674557 |
| Antibody detection by ELISA +++ | Bulk milk, blood | DSe and DSp may differdepending on the ELISA used (commercial/in-house) and the antibodies being tested (e.g. antibodies against structural (E2) and non-structural (NS2-3) proteins. | | | - Simple to perform and cost-effective<br>- Milk collection is non-invasive method | - Some cross-reactivity with antibodies induced by vaccines and other pestiviruses.<br>- PI animal will be seronegative<br>- Bulk milk from herd excludes males, non-lactating or young stock. | Barrett *et al.* (2022) *BMC Vet Res.*, **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 | - Very high specificity<br>- Allows differentiation of antibodies to BVDV species | - ASe can vary depending on virus strain used<br>- Requires cell culture, good quality samples<br>- Takes 5 days to obtain results. Labour intensive - not | N/A |

| 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 |
|---|---|---|---|---|---|---|---|
| | | | | | | amenable to testing very large numbers of samples,<br>- No differentiation between infected and vaccinated animals | |

16 N/A: not available

17 **Appendix 6: Bovine viral diarrhoea**

18 **Intended purpose of test: immune status in individual animals or populations (post-vaccination)**

| **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** |
|---|---|---|---|---|---|---|---|
| Antibody detection by ELISA +++ | Individual milk, bulk milk, blood (antibodies present against structural and non-structural proteins) | 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. | | | - Simple to perform and cost-effective<br>- Milk collection is non-invasive method | - Some cross-reactivity with antibodies induced by vaccines and other pestiviruses.<br>While a DIVA capability is preferred, this is very difficult 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.<br>- PI animal will be seronegative<br>- Bulk milk from herd excludes males, non-lactating or young stock | Raue *et al.* (2011). *Vet. J.*, 187, 330–334; Gonzalez *et al.* (2014). *Vet J.*, **199**, 424–428. Sayers *et al.*, (2015). *Vet. J.*, **205**, 56–61. |
| Virus neutralisation test +++ | Serum | DSe & DSp both extremely high, both >99%. Historical reference serological test. | N/A | Historical information with no formal validation | - Very high specificity<br>- Good correlation with immunity<br>- Can provide a measure of cross protection between BVDV species | - ASe can vary depending on virus strain used<br>- Requires cell culture, good quality samples<br>- Labour intensive, takes 5 days to obtain results<br>- No differentiation between infected and vaccinated animals | N/A |

19 N/A: not available

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# 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.*

***Requirements for vaccines:*** *All strains of capripoxvirus examined so far, whether derived from cattle, sheep or goats, are antigenically similar. Attenuated cattle strains, and strains derived from sheep and goats have been used as live vaccines against LSDV.*

# A. INTRODUCTION

Lumpy skin disease (LSD) was first seen in Zambia in 1929, spreading into Botswana by 1943 (Haig, 1957), and then into South Africa the same year, where it affected over eight million cattle causing major economic loss. In 1957 it entered Kenya, ~~at the same time as~~ associated with an outbreak of sheep pox (Weiss, 1968). In 1970 LSD spread north into the Sudan, by 1974 it had spread west as far as Nigeria, and in 1977 was reported from Mauritania, Mali, Ghana and Liberia. Another epizootic of LSD between 1981 and 1986 affected Tanzania, Kenya, Zimbabwe, Somalia and the Cameroon, with reported mortality rates in affected cattle of 20%. The occurrence of LSD north of the Sahara desert and outside the African continent was confirmed for the first time in Egypt and Israel between 1988 and 1989, and was reported again in 2006 (Brenner *et al.,* 2006). In the past decade, LSD occurrences have been reported in the Middle Eastern, European and Asian regions (for up-to-date information, consult WOAH WAHIS interface[1]). Lumpy skin disease outbreaks tend to be sporadic, depending upon animal movements, immune status, and wind and rainfall patterns affecting vector populations. The principal method of transmission is thought to be mechanical by various arthropod vectors (Tuppurainen *et al*., 2015).

Lumpy skin disease virus (LSDV) belongs to the family *Poxviridae*, subfamily *Chordopoxvirinae* ~~*Chordopoxviridae*~~, and genus *Capripoxvirus*. In common with other poxviruses LSDV replicates in the cytoplasm of an infected cell, forming distinct perinuclear viral factories. The LSD virion is large and brick-shaped measuring 293–299nm (length) and 262–273nm (width). The LSDV genome structure is also similar to other poxviruses, consisting of double-stranded linear DNA that is 25% GC-rich, approximately 150,000 bp in length, and encodes around 156 open reading frames (ORFs). An inverted terminal repeat sequence of 2200–2300 bp is found at each end of the linear genome. The linear ends of the genome are joined with a hairpin loop. The central region of the LSDV genome contains ORFs predicted to encode proteins required for virus replication and morphogenesis and exhibit a high degree of similarity with genomes of other mammalian poxviruses. The ORFs in the outer regions of the LSDV genome have lower similarity and likely encode proteins involved in viral virulence and host range determinants.

Phylogenetic analysis shows the majority of LSDV strains group into two monophyletic clusters (cluster 1.1 and 1.2) (Biswas *et al.,* 2020; Van Schalkwyk *et al.,* 2021). Cluster 1.1 consists of LSDV Neethling vaccine strains that are based on the LSDV/Neethling/LW-1959 vaccine strain (Kara *et al.,* 2003; Van Rooyen *et al.,* 1959; van Schalkwyk *et al.,* 2020) and historic wild-type strains from South Africa. Cluster 1.2 consists of wild-type strains from southern Africa, Kenya, the northern hemisphere, and the Kenyan KSGP O-240 commercial vaccine. In addition to these two clusters, there have recently been recombinant LSDV strains isolated from clinical cases of LSD in the field in Russia and central Asia (Flannery *et al.,* 2021; Sprygin *et al.,* 2018; 2020; Wang *et al.,* 2021). These recombinant viruses show unique patterns of accessory gene alleles, consisting of sections of both wild-type and "vaccine" LSDV strains.

The severity of the clinical signs of LSD is highly variable and depends on a number of factors, including the strain of capripoxvirus, the age of the host, immunological status and breed. *Bos taurus* is generally more susceptible to clinical disease than *Bos indicus;* the Asian buffalo (*Bubalus spp.*) has also been reported to be susceptible. Within *Bos taurus,* the fine-skinned Channel Island breeds develop more severe disease, with lactating cows appearing to be the most at risk. However, even among groups of cattle of the same breed kept together under the same conditions, there is a large variation in the clinical signs presented, ranging from subclinical infection to death (Carn & Kitching, 1995). There may be failure of the virus to infect the whole group, probably depending on the virulence of the virus isolate, immunological status of the host, host genotype~~,~~ and vector prevalence. Seroprevalence studies, experimental infections and case reports have provided indications that several wildlife species (e.g. springbok, impala, giraffe, camel, banteng) are susceptible to LSDV infection (Dao *et al.,* 2022; Hedger & Hamblin, 1983; Kumar *et al.,* 2023; Porco *et al.,* 2023). The scarcity of documented outbreaks in wildlife and the fact that available studies remain limited in number and mostly involve only a few animals, make it difficult to determine the role of wildlife in LSDV epidemiology. This topic deserves further study, especially given the current spread of LSDV in new geographical areas where large numbers of naïve, potentially susceptible wild bovines and other ruminants are present.

The incubation period under field conditions has not been reported, but following experimental inoculation is 6–9 days until the onset of fever. In the acutely infected animal, there is an initial pyrexia, which may exceed 41°C and persist for 1 week. All the superficial lymph nodes become enlarged. In lactating cattle there is a marked reduction in milk yield. Lesions develop over the body, particularly on the head, neck, udder, scrotum, vulva and perineum between 7 and 19 days after virus inoculation (Coetzer, 2004). The characteristic integumentary lesions are multiple, well circumscribed to coalescing, 0.5–5 cm in diameter, firm, flat-topped papules and nodules. The nodules involve the dermis and epidermis, and may

[1] https://www.woah.org/en/what-we-do/animal-health-and-welfare/disease-data-collection/

90 extend to the underlying subcutis and occasionally to the adjacent striated muscle. These nodules have a creamy grey to
91 white colour on cut section, which may initially exude serum, but over the ensuing 2 weeks a cone-shaped central core or
92 sequestrum of necrotic material/necrotic plug ("sit-fast") may appear within the nodule. The acute histological lesions
93 consist of epidermal vacuolar changes with intracytoplasmic inclusion bodies and dermal vasculitis. The inclusion bodies
94 are numerous, intracytoplasmic, eosinophilic, homogenous to occasionally granular and they may occur in endothelial
95 cells, fibroblasts, macrophages, pericytes, and keratinocytes. The dermal lesions include vasculitis with fibrinoid necrosis,
96 oedema, thrombosis, lymphangitis, dermal-epidermal separation, and mixed inflammatory infiltrate. The chronic lesions
97 are characterised by an infarcted tissue with a sequestered necrotic core, often rimmed by granulation tissue gradually
98 replaced by mature fibrosis. At the appearance of the nodules, the discharge from the eyes and nose becomes
99 mucopurulent, and keratitis may develop. Nodules may also develop in the mucous membranes of the mouth and
100 alimentary tract, particularly the abomasum and in the trachea and the lungs, resulting in primary and secondary
101 pneumonia. The nodules on the mucous membranes of the eyes, nose, mouth, rectum, udder and genitalia quickly
102 ulcerate, and by then all secretions, ocular and nasal discharge and saliva contain LSD virus (LSDV). The limbs may be
103 oedematous and the animal is reluctant to move. Pregnant cattle may abort, and there is a report of intrauterine
104 transmission (Rouby & Aboulsoudb, 2016). Bulls may become permanently or temporarily infertile and the virus can be
105 excreted in the semen for prolonged periods (Irons *et al*., 2005). Recovery from severe infection is slow; the animal is
106 emaciated, may have pneumonia and mastitis, and the necrotic plugs of skin, which may have been subject to fly strike,
107 are shed leaving deep holes in the hide (Prozesky & Barnard, 1982).

108 The main differential diagnosis is pseudo-LSD caused by bovine herpesvirus 2 (BoHV-2). This is usually a milder clinical
109 condition, characterised by superficial nodules, resembling only the early stage of LSD. Intra-nuclear inclusion bodies and
110 viral syncytia are histopathological characteristics of BoHV-2 infection not seen in LSD. Other differential diagnoses (for
111 integumentary lesions) include: dermatophilosis, dermatophytosis, bovine farcy, photosensitisation, actinomycosis,
112 actinobacillosis, urticaria, insect bites, besnoitiosis, nocardiosis, demodicosis, onchocerciasis, pseudo-cowpox, and
113 cowpox. Differential diagnoses for mucosal lesions include: foot and mouth disease, bluetongue, mucosal disease,
114 malignant catarrhal fever, infectious bovine rhinotracheitis, and bovine papular stomatitis.

115 LSDV is not transmissible to humans. However, all laboratory manipulations must be performed at an appropriate
116 containment level determined using biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing*
117 *biological risk in the veterinary laboratory and animal facilities*).

118 # B. DIAGNOSTIC TECHNIQUES

119 ***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 | + | ++ | + | +++ | + | – |
| PCR | ++ | +++ | ++ | +++ | + | – |
| TEM | – | – | – | + | – | – |
| Detection of immune response | | | | | | |
| VNT | ++ | ++ | ++ | ++ | ++ | ++ |
| IFAT | + | + | + | + | + | + |
| ELISA | ++ | ++ | ++ | ++ | ++ | ++ |

120 Key: +++ = recommended for this purpose; ++ recommended but has limitations;
121 + = suitable in very limited circumstances; – = not appropriate for this purpose.
122 PCR = polymerase chain reaction; TEM = Transmission electron microscopy; VNT = virus neutralisation test;
123 IFAT = indirect fluorescent antibody test; ELISA = enzyme-linked immunosorbent assay.

124 ## 1. Detection of the agent

125 ### 1.1. Specimen collection, submission and preparation

126 Material for virus isolation and antigen detection should be collected as biopsies or from skin nodules at post-
127 mortem examination. Samples for virus isolation should preferably be collected within the first week of the
128 occurrence of clinical signs, before the development of neutralising antibodies (Davies, 1991; Davies *et al*.,
129 1971), however virus can be isolated from skin nodules for at least 3–4 weeks thereafter. Samples for genome
130 detection using conventional or real-time polymerase chain reaction (PCR) may be collected when neutralising
131 antibody is present. Following the first appearance of the skin lesions, the virus can be isolated for up to 35 days
132 and viral nucleic acid can be demonstrated via PCR for up to 3 months (Tuppurainen *et al*., 2005; Weiss, 1968).
133 Buffy coat from blood collected into heparin or EDTA (ethylene diamine tetra-acetic acid) during the viraemic
134 stage of LSD (before generalisation of lesions or within 4 days of generalisation), can also be used for virus
135 isolation. Samples for histology should include the lesion and tissue from the surrounding (non-lesion) area, be
136 a maximum size of 2 $cm^3$, and be placed immediately following collection into ten times the sample volume of
137 10% neutral buffered formal saline.

138 Tissues in formalin have no special transportation requirements in regard to biorisks. Blood samples with
139 anticoagulant for virus isolation from the buffy coat should be placed immediately on ice after gentle mixing and
140 processed as soon as possible. In practice, the samples may be kept at 4°C for up to 2 days prior to processing,
141 but should not be frozen or kept at ambient temperatures. Tissues for virus isolation and antigen detection
142 should be kept at 4°C, on ice or at –20°C. If it is necessary to transport samples over long distances without
143 refrigeration, the medium should contain 10% glycerol; the samples should be of sufficient size (e.g. 1 g in 10 ml)
144 that the transport medium does not penetrate the central part of the biopsy, which should be used for virus
145 isolation.

146 ~~Samples for histology should include the lesion and tissue from the surrounding (non-lesion) area, be a~~
147 ~~maximum size of 2 cm³, and be placed immediately following collection into ten times the sample volume of~~
148 ~~10% neutral buffered formaldehyde. Tissues in formalin have no special transportation requirements in regard~~
149 ~~to biorisks.~~ Material for histology should be prepared using standard techniques and stained with haematoxylin
150 and eosin (H&E) (Burdin, 1959). Lesion material for virus isolation and antigen detection is minced using a sterile
151 scalpel blade and forceps and then macerated in a sterile steel ball-bearing mixer mill, or ground with a pestle
152 in a sterile mortar with sterile sand and an equal volume of sterile phosphate buffered saline (PBS) or serum-
153 free modified Eagle's medium containing sodium penicillin (1000 international units [IU]/ml), streptomycin
154 sulphate (1 mg/ml), mycostatin (100 IU/ml) or fungizone (amphotericin, 2.5 µg/ml) and neomycin (200 IU/ml).
155 The suspension is freeze–thawed three times and then partially clarified using a bench centrifuge at 600 ***g*** for
156 10 minutes. In cases where bacterial contamination of the sample is expected (such as when virus is isolated
157 from skin samples), the supernatant can be filtered through a 0.45 µm pore size filter after the centrifugation
158 step. Buffy coats may be prepared from unclotted blood using centrifugation at 600 ***g*** for 15 minutes, and the
159 buffy coat carefully removed into 5 ml of cold double-distilled water using a sterile Pasteur pipette. After
160 30 seconds, 5 ml of cold double-strength growth medium is added and mixed. The mixture is centrifuged at
161 600 ***g*** for 15 minutes, the supernatant is discarded and the cell pellet is suspended in 5 ml growth medium, such
162 as Glasgow's modified Eagle's medium (GMEM). After centrifugation at 600 ***g*** for a further 15 minutes, the
163 resulting pellet is suspended in 5 ml of fresh GMEM. Alternatively, the buffy coat may be separated from a
164 heparinised sample by using a Ficoll gradient.

165 ### 1.2. Virus isolation on cell culture

166 LSDV will grow in tissue culture of bovine, ovine or caprine origin. MDBK (Madin–Darby bovine kidney) cells are
167 often used, as they support good growth of the virus and are well characterised (Fay *et al.,* 2020). Primary cells,
168 such as lamb testis (LT) cells also support viral growth, but care needs to be taken to ensure they are not
169 contaminated with viruses such as bovine viral diarrhoea virus. One ml of clarified supernatant or buffy coat is
170 inoculated onto a confluent monolayer in a 25 $cm^2$ culture flask at 37°C and allowed to adsorb for 1 hour. The
171 culture is then washed with warm PBS and covered with 10 ml of a suitable medium, such as GMEM, containing
172 antibiotics and 2% fetal calf serum. If available, tissue culture tubes containing appropriate cells and a flying
173 cover-slip, or tissue culture microscope slides, are also infected.

174 The flasks/tissue culture tubes are examined daily for 7–14 days for evidence of cytopathic effects (CPE).
175 Infected cells develop a characteristic CPE consisting of retraction of the cell membrane from surrounding cells,
176 and eventually rounding of cells and margination of the nuclear chromatin. At first only small areas of CPE can
177 be seen, sometimes as soon as 2 days after infection; over the following 4–6 days these expand to involve the
178 whole cell monolayer ~~sheet~~. If no CPE is apparent by day 14, the culture should be freeze–thawed three times,
179 and clarified supernatant inoculated on to a fresh cell monolayer. At the first sign of CPE in the flasks, or earlier
180 if a number of infected cover-slips are being used, a cover-slip should be removed, fixed in acetone and stained

using H&E. Eosinophilic intracytoplasmic inclusion bodies, which are variable in size but up to half the size of the nucleus and surrounded by a clear halo, are diagnostic for poxvirus infection. PCR may be used as an alternative to H&E for confirmation of the diagnosis. The CPE can be prevented or delayed by adding specific anti-LSDV serum to the medium. In contrast, the herpesvirus that causes pseudo-LSD produces a Cowdry type A intranuclear inclusion body. It also forms syncytia.

An ovine testis cell line (OA3.T~~s~~) has been evaluated for the propagation of capripoxvirus isolates (Babiuk *et al.*, 2007), however this cell line has been found to be contaminated with pestivirus and should be used with caution.

### 1.3. Polymerase chain reaction (PCR)

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).

#### 1.3.1. Test procedure

The extraction method described below can be replaced using commercially available DNA extraction kits.

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.

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.

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 –20°C (Tuppurainen *et al.*, 2005). Alternatively a column-based extraction kit may be used.

iv) The primers for this PCR assay were developed from the gene encoding the viral attachment protein. The size of the expected amplicon is 192 bp (Ireland & Binepal, 1998). The primers have the following gene sequences:

Forward primer 5’-TCC-GAG-CTC-TTT-CCT-GAT-TTT-TCT-TAC-TAT-3’

Reverse primer 5’-TAT-GGT-ACC-TAA-ATT-ATA-TAC-GTA-AAT-AAC-3’.

v) DNA amplification is carried out in a final volume of 50 µl containing: 5 µl of 10 × PCR buffer, 1.5 µl of $MgCl_2$ (50 mM), 1 µl of dNTP (10 mM), 1 µl of forward primer, 1 µl of reverse primer, 1 µl of DNA template (~10 ng), 0.5 µl of *Taq* DNA polymerase and 39 µl of nuclease-free water. The volume of DNA template required may vary and the volume of nuclease-free water must be adjusted to the final volume of 50 µl.

vi) Run the samples in a thermal cycler as follows: 2 minutes at 95°C; then 45 seconds at 95°C, 50 seconds at 50°C and 1 minute at 72°C (34 cycles); 2 minutes at 72°C and hold at 4°C until analysis.

vii) Mix 10 µl of each sample with loading dye and load onto a 1.5% agarose gel in TAE buffer (Tris/acetate buffer containing EDTA). Load a parallel lane with a 100 bp DNA-marker ladder. Electrophoretically separate the products using approximately 8–10 V/cm for 40–60 minutes and visualise with a suitable DNA stain and transilluminator.

Quantitative real-time PCR methods have been described that are reported to be faster and have higher sensitivity than conventional PCRs (Balinsky *et al.*, 2008; Bowden *et al.*, 2008). A real-time PCR method that differentiates between LSDV, sheep pox virus and goat pox virus has been published (Lamien *et al.,* 2011).

Quantitative real-time PCR assays have been designed to differentiate between Neethling-based LSDV strains, which are often used for vaccination, and wild-type LSDV strains from cluster 1.2 (Agianniotaki *et al.,* 2017;

Pestova *et al.,* 2018; Vidanovic *et al.,* 2016). These "DIVA" assays (DIVA: differentiation of infected from vaccinated animals) enable, for example, differentiation of "Neethling response" caused by vaccination with a LSDV Neethling vaccine strain from disease caused by infection with a cluster 1.2 wild-type virus. However these DIVA PCR assays cannot distinguish between a LSDV Neethling vaccine strain and the novel recombinant LSDV strains ~~recently~~ isolated from disease outbreaks in Asia (~~Byadovskaya *et al.,* 2021;~~ Flannery *et al.,* 2021). These DIVA assays are also not capable of discriminating between LSDV Neethling vaccine strains and recently characterised (historic) wild-type viruses from South Africa belonging within cluster 1.1 (Van Schalkwyk *et al.,* 2020; 2021). Consequently, in regions where recombinant strains (currently Asia and possibly elsewhere) or wild-type cluster 1.1 strains are circulating (currently South Africa and possibly elsewhere), these DIVA assays are not suitable for distinguishing vaccine and wild-type virus. Thus, in order to overcome these constraints, whole genome sequencing is recommended.

## 1.4. Transmission electron microscopy

The characteristic poxvirus virion can be visualised using a negative staining preparation technique followed by examination with an electron microscope. There are many different negative staining protocols, an example of which is given below.

### 1.4.1. Test procedure

Before centrifugation, material from the original biopsy suspension is prepared for examination under the transmission electron microscope by floating a 400-mesh hexagonal electron microscope grid, with pioloform-carbon substrate activated by glow discharge in pentylamine vapour, onto a drop of the suspension placed on parafilm or a wax plate. After 1 minute, the grid is transferred to a drop of Tris/EDTA buffer, pH 7.8, for 20 seconds and then to a drop of 1% phosphotungstic acid, pH 7.2, for 10 seconds. The grid is drained using filter paper, air-dried and placed in the electron microscope. The capripox virion is brick shaped, covered in short tubular elements and measures approximately 290 × 270 nm. A host-cell-derived membrane may surround some of the virions, and as many as possible should be examined to confirm their appearance (Kitching & Smale, 1986).

The ~~capripox~~ virions of capripoxvirus are indistinguishable from those of orthopoxvirus, but, apart from vaccinia virus and cowpox virus, which are both uncommon in cattle and do not cause generalised infection, no other orthopoxvirus is known to cause lesions in cattle. However, vaccinia virus may cause generalised infection in young immunocompromised calves. In contrast, orthopoxviruses are a common cause of skin disease in domestic buffalo (*Bubalus bubalis*) causing buffalo pox, a disease that usually manifests as pock lesions on the teats, but may cause skin lesions at other sites, such as the perineum, the medial aspects of the thighs and the head. Orthopoxviruses that cause buffalo pox cannot be readily distinguished from capripoxvirus by electron microscopy. The virions of parapoxvirus ~~virions~~ that cause bovine papular stomatitis and pseudocowpox are smaller, oval in shape and each is covered in a single continuous tubular element that appears as striations over the virion. Capripoxvirus virions are also distinct from the herpesvirus that causes pseudo-LSD (also known as "Allerton" or bovine herpes mammillitis).

## 1.5. Fluorescent antibody tests

Capripoxvirus antigen can 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 cattle sera is subject to high background colour and nonspecific reactions. However, a direct conjugate can be prepared from sera from convalescent cattle (or from sheep or goats convalescing from capripox) or from rabbits hyperimmunised with purified capripoxvirus. Uninfected tissue culture should be included as a negative control as cross-reactions can cause problems due to antibodies to cellular components (pre-absorption of these from the immune serum helps solve this issue).

## 1.6. Immunohistochemistry

Immunohistochemistry using F80G5 monoclonal antibody specific for capripoxvirus ORF 057 has been described for detection of LSDV antigen in the skin of experimentally infected cattle (Babiuk *et al.,* 2008).

## 1.7. Isothermal genome amplification

Molecular tests using loop-mediated isothermal amplification to detect capripoxvirus genomes are reported to provide sensitivity and specificity similar to real-time PCR with a simpler method and lower cost (Das *et al.,* 2012; Murray *et al.,* 2013). Field validation of the Das *et al.* method was reported by Omoga *et al.* (2016).

## 2. Serological tests

All the viruses in the genus *Capripoxvirus* share a common major antigen for neutralising antibodies and it is thus not possible to distinguish strains of capripoxvirus from cattle, sheep or goats using serological techniques.

### 2.1. Virus neutralisation

A test serum can either be titrated against a constant titre of capripoxvirus (100 $TCID_{50}$ [50% tissue culture infective dose]) or a standard virus strain can be titrated against a constant dilution of test serum in order to calculate a neutralisation index. Because of the variable sensitivity of tissue culture to capripoxvirus, and the consequent difficulty of ensuring the accurate and repeatable seeding of 100 $TCID_{50}$/well, the neutralisation index is the preferred method in most laboratories, although it does require a larger volume of test sera. The test is described using 96-well flat-bottomed tissue-culture grade microtitre plates, but it can be performed equally well in tissue culture tubes with the appropriate changes to the volumes used, although it is more difficult to read an end-point in tubes.

#### 2.1.1. Test procedure

i) Test sera, including a negative and a positive control, are diluted 1/5 in Eagle's/HEPES (N-2-hydroxyethylpiperazine, N-2-ethanesulphonic acid) buffer and inactivated at 56°C for 30 minutes.

ii) Next, 50 µl of the first inactivated serum is added to columns 1 and 2, rows A to H of the microtitre plate. The second serum is placed in columns 3 and 4, the third in columns 5 and 6, the positive control serum is placed in columns 7 and 8, the negative control serum is placed in columns 9 and 10, and 50 µl of Eagle's/HEPES buffer (without serum) is placed in columns 11 and 12, and to all wells in row H.

iii) A reference strain of capripoxvirus, usually a vaccine strain known to grow well in tissue culture, with a titre of over $\log_{10}$ 6 $TCID_{50}$ per ml is diluted in Eagle's/HEPES in bijoux bottles to give a log dilution series of $\log_{10}$ 5.0, 4.0, 3.5, 3.0, 2.5, 2.0, 1.5 $TCID_{50}$ per ml (equivalent to $\log_{10}$ 3.7, 2.7, 2.2, 1.7, 1.2, 0.7, 0.2 $TCID_{50}$ per 50 µl).

iv) Starting with row G and the most diluted virus preparation, 50 µl of virus is added to each well in that row. This is repeated with each virus dilution, the highest titre virus dilution being placed in row A.

v) The plates are covered and incubated for 1 hour at 37°C.

vi) An appropriate cell suspension (such as MDBK cells) is prepared from pregrown monolayers as a suspension of $10^5$ cells/ml in Eagle's medium containing antibiotics and 2% fetal calf serum. Following incubation of the microtitre plates, 100 µl of cell suspension is added to all the wells, except wells H11 and H12, which serve as control wells for the medium. The remaining wells of row H are cell and serum controls.

vii) The microtitre plates are covered and incubated at 37°C for 9 days.

viii) Using an inverted microscope, the monolayers are examined daily from day 4 for evidence of CPE. There should be no CPE in the cells of row H. Using the 0240 KSGP vaccine strain of capripoxvirus, by way of example, the final reading is taken on day 9, and the titre of virus in each duplicate titration is calculated using the Kärber method. If left longer, there is invariably a 'breakthrough' of virus in which virus that was at first neutralised appears to disassociate from the antibody.

ix) *Interpretation of the results:* The neutralisation index is the log titre difference between the titre of the virus in the negative serum and in the test serum. An index of ≥1.5 is positive. The test can be made more sensitive if serum from the same animal is examined before and after infection. Because the immunity to capripoxviruses is predominantly cell mediated, a negative result, particularly following vaccination, after which the antibody response may be low, does not imply that the animal from which the serum was taken is not protected.

Antibodies to capripoxvirus can be detected from 1 to 2 days after the onset of clinical signs. These remain detectable for about 7 months.

### 2.2. Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assays (ELISAs) for the detection of capripoxviral antibodies are widely used and are available in commercial kit form (Milovanovic *et al.,* 2019; Samojlovic *et al.,* 2019).

### 2.3. Indirect fluorescent antibody test

Capripoxvirus-infected tissue culture grown on cover-slips or tissue culture microscope slides can be used for the indirect fluorescent antibody test. Uninfected tissue culture control, and positive and negative control sera, should be included in the test. The infected and control cultures are fixed in acetone at –20°C for 10 minutes and stored at 4°C. Dilutions of test sera are made in PBS, starting at 1/20 or 1/40, and positive samples are identified using an anti-bovine gamma-globulin conjugated with fluorescein isothiocyanate. Antibody titres may exceed 1/1000 after infection. Sera may be screened at 1/50 and 1/500. Cross-reactions can occur with orf virus (contagious pustular dermatitis virus of sheep), bovine papular stomatitis virus and perhaps other poxviruses.

### 2.4. Western blot analysis

Western blotting of test sera against capripoxvirus-infected cell lysate provides a sensitive and specific system for the detection of antibody to capripoxvirus structural proteins, although the test is expensive and difficult to carry out.

Capripoxvirus-infected LT cells should be harvested when 90% CPE is observed, freeze–thawed three times, and the cellular debris pelleted using centrifugation. The supernatant should be decanted, and the proteins should be separated using SDS/PAGE (sodium dodecyl sulphate/polyacrylamide gel electrophoresis). A vertical discontinuous gel system, using a stacking gel made up of acrylamide (5%) in Tris (125 mM), pH 6.8, and SDS (0.1%), and a resolving gel made up of acrylamide (10–12.5%) in Tris (560 mM), pH 8.7, and SDS (0.1%), is recommended for use with a glycine running buffer containing Tris (250 mM), glycine (2 M), and SDS (0.1%). Samples of supernatant should be prepared by boiling for 5 minutes with an appropriate lysis buffer prior to loading. Alternatively, purified virus or recombinant antigens may replace tissue-culture-derived antigen.

Molecular weight markers should be run concurrently with the protein samples. The separated proteins in the SDS/PAGE gel should be transferred electrophoretically to a nitrocellulose membrane (NCM). After transfer, the NCM is rinsed thoroughly in PBS and blocked in 3% bovine serum albumin (BSA) in PBS, or 5% skimmed milk powder in PBS, on a rotating shaker at 4□C overnight. The NCM can then be separated into strips by employing a commercial apparatus to allow the concurrent testing of multiple serum samples, or may be cut into strips and each strip incubated separately thereafter. The NCM is washed thoroughly with five changes of PBS for 5 minutes on a rotating shaker, and then incubated at room temperature on the shaker for 1.5 hours, with the appropriate serum at a dilution of 1/50 in blocking buffer (3% BSA and 0.05% Tween 20 in PBS; or 5% milk powder and 0.05% Tween 20 in PBS). The membrane is again thoroughly washed and incubated (in blocking buffer) with anti-species immunoglobulin horseradish-peroxidase-conjugated immunoglobulins at a dilution determined using titration. After further incubation at room temperature for 1.5 hours, the membrane is washed and a solution of diaminobenzidine tetrahydrochloride (10 mg in 50 ml of 50 mM ~~mm~~ Tris/HCl, pH 7.5, and 20 µl of 30% [v/v] hydrogen peroxide) is added. Incubation is then undertaken for approximately 3–7 minutes at room temperature on a shaker with constant observation, and the reaction is stopped by washing the NCM in PBS before excessive background colour is seen. A positive and negative control serum should be used on each occasion.

Positive test samples and the positive control will produce a pattern consistent with reaction to proteins of molecular weights 67, 32, 26, 19 and 17 kDa – the major structural proteins of capripoxvirus – whereas negative serum samples will not react with all these proteins. Hyperimmune serum prepared against parapoxvirus (bovine papular stomatitis or pseudocowpox virus) will react with some of the capripoxvirus proteins, but not the 32 kDa protein that is specific for capripoxvirus.

# C. REQUIREMENTS FOR VACCINES

## 1. Background: rationale and intended use of the product

~~Live attenuated strains of capripoxvirus have been used as vaccines specifically for the control of LSD (Brenner *et al*., 2006; Capstick & Coakley, 1961; Carn, 1993). Capripoxviruses are cross-reactive within the genus. Consequently, it is possible to protect cattle against LSD using strains of capripoxvirus derived from sheep or goats (Coakley & Capstick, 1961). However, it is recommended to carry out controlled trials, using the most susceptible breeds, prior to introducing a vaccine strain not usually used in cattle. The duration of protection provided by LSD vaccination is unknown.~~

~~Capripoxvirus vaccine strains can produce a large local reaction at the site of inoculation in *Bos taurus* breeds (Davies, 1991), which some stock owners find unacceptable. This has discouraged the use of vaccine, even though the consequences of an outbreak of LSD are invariably more severe. Risk–benefit of vaccination should be assessed following stakeholder discussion.~~

Vaccines are a key tool to control LSD. Different types of LSD vaccines have been developed and several are commercially available (Tuppurainen *et al.,* 2021).

Live attenuated vaccines (LAV) based on the Neethling LSDV strain (homologous LAV vaccines) have been shown to offer high levels of protection against LSD under experimental conditions (Haegeman *et al.,* 2021) and have been used successfully to control the disease in the field, through systematic vaccination of the entire country's cattle population for a number of consecutive years (Klement *et al.,* 2020). Homologous vaccines may induce fever, produce a local reaction at the site of inoculation, cause a temporary reduction in milk production and on rare occasions induce a 'Neethling' response (Ben-Gera *et al.,* 2015; Davies, 1991; Haegeman *et al.,* 2021). Such adverse effects, however, usually resolve within a few days and are largely outweighed by the overall benefits of vaccination with homologous vaccines. The duration of immunity induced by good quality live attenuated LSDV vaccines was shown to be at least 18 months (Haegeman *et al.,* 2023).

As capripoxviruses provide cross-reactive protection within the genus, heterologous LAVs comprising sheeppox virus or goatpox virus strains have also been tested and used to protect cattle against LSD. Sheeppox virus-based heterologous vaccines usually contain higher doses of virus than when administered to sheep. Although safe, their effectiveness in protecting cattle against LSD is inferior compared to homologous vaccines (Ben-Gera *et al*., 2015; Zhugunissov *et al*., 2020). Heterologous vaccines containing goatpox virus strains for use in cattle against LSD have been developed more recently. One such vaccine based on the Gorgan strain provided protection under experimental conditions comparable to homologous vaccines (Gari *et al*., 2015). On the other hand, a goat pox vaccine based on an attenuated Uttarkashi goatpox virus strain performed suboptimally under field conditions in India (Naveem *et al*., 2023), indicating that further research is warranted before asserting that all goatpox virus-based vaccines induce protection equal to homologous vaccines in cattle against LSD.

In addition, homologous inactivated vaccines against LSD have been developed and tested (Haegeman *et al*., 2023; Hamdi *et al*., 2020; Wolff *et al*., 2022). These vaccines are reported to be safe and efficacious. They however require a booster vaccination one month after primo-vaccination and then every 6 months thereafter, based on the fact that the duration of immunity is shorter than 1 year (Haegeman *et al*., 2023).

None of the commercial vaccines currently available has practical DIVA capacity. This problem may be resolved in the future by introducing new types of vaccines (e.g. vector-vaccines, subunit vaccines, mRNA vaccines) that are at various stages of development and evaluation.

## 2. Outline of production of LSD vaccines ~~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 standard operating procedures (SOP) for the method of manufacture and each step for the testing of cells and reagents used in the process, each batch and the final product.~~

The production of vaccines, including LSD vaccines, starts within research and development (R&D) facilities where vaccine candidates are produced and tested in preclinical studies to demonstrate the quality, safety and efficacy of the product.

Minimum requirements for different production stages of veterinary vaccines are available in different chapters of the *Terrestrial Manual.* These are intended to be used in combination with country-specific regulatory requirements for vaccine production and release. Here we outline the most important requirements for the production of live and inactivated LSD vaccines. Full requirements are available in Chapter 1.1.8 *Principles of veterinary vaccine production,* Chapter 2.3.3 *Minimum requirements for the organisation and management of a vaccine manufacturing facility* and Chapter 2.3.4 *Minimum requirements for the production and quality control of vaccine,* and other regulatory documentation.

### 2.1. Quality assurance

Facilities for manufacturing LSD vaccines should operate in line with the concepts of good laboratory practice (GLP) and good manufacturing practice (GMP) to produce high quality products. Quality risk management and quality control with adequate documentation management, as an integral part of the production process, have to be in place. In case some activities of the production process are outsourced, those should also be appropriately defined, recorded and controlled.

The vaccine production process (Outline of Production) should be documented in a series of standard operating procedures (SOPs), or other documents describing the manufacturing of each batch and the final product (including starting materials to be used, manufacturing steps, in-process controls and controls on the final

product). Detailed requirements for documentation management in the process of vaccine production are available in Chapter 2.3.3.

A completed Outline of Production is to be enclosed in a vaccine candidate dossier and used for the evaluation of the production process and product by regulatory bodies.

### 2.2. Process validation

The dossier with the enclosed Outline of Production for the vaccine candidate has to be submitted for regulatory approval, so it can be assessed and authorised by the competent authority to ensure compliance with local regulatory requirements. Among others, data on quality, safety, and efficacy will be assessed. The procedures necessary to obtain these data are described in the subsequent sections.

National regulatory authorities might also require official control authority re-testing (check testing) of final products and batches in government laboratories or an independent batch quality control by a third party.

## 3. Requirements for LSD vaccine candidates and batch production

### 3.1. Requirements for starting materials

Live attenuated vaccines (LAV) and inactivated vaccines (IV) for LSD are produced using the system of limited and controlled passages of master seed and working seed virus and cell banks with a specified maximum. This approach aims to prevent possible and unwanted drift of properties of seed virus and cells that might arise from repeated passaging.

#### 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 in one or more (but, limited) cell culture passages from the master seed stock and used to produce vaccine batches. This approach and limitation of seed virus passaging will assist in maintaining uniformity and consistency in production.

#### 3.1.2. Master cell stocks

The production process of LSD vaccines ideally employs an established master cell stock (MCS) system with defined lowest and highest cell passage to be used to grow the vaccine virus. Primary cells derived from normal tissues can be used in the production process, but the use of primary cells has an inherently higher risk of introducing extraneous agents compared with the use of established (well characterised) cell lines and should be avoided where alternative methods of producing effective vaccines exist. For each MCS, manufacturers should demonstrate:

- MCS identity
- genetic stability by subculturing from the lowest to the highest passage used for production
- stable MCS karyotype with a low level of polyploidy
- freedom from oncogenicity or tumorigenicity by using *in-vivo* studies using the highest cell passage that may be used for production
- purity of MCSs from extraneous bacteria, fungi, mycoplasma, and viruses
- implemented measures to lower TSE contamination risk (see Section C.3.5.1 *Purity tests*).

### 3.2. Method of vaccine manufacturing

~~The method of manufacture should be documented as the Outline of Production.~~

#### ~~2.2.1. Procedure~~

#### 3.2.1. LSD vaccine batch production

~~Vaccine batches are produced on an appropriate cell line such as MDBK.~~ As already mentioned in the first paragraphs of Section C, all steps undertaken in the production of vaccine batches should be described and documented in the Outline of Production. The production of LAV and IV against LSD starts with the inoculation of the required number of working vials of seed virus ~~is~~ reconstituted ~~with GMEM or other~~ in appropriate medium ~~and inoculated~~ onto a suitable primary or continuous cell line grown in suspension or monolayer~~. Cells should be harvested after 4–8 days when they exhibit 50–70% CPE for maximum~~ in the exponential growth phase. At the time highest viral ~~infectivity, or earlier if CPE is extensive and cells appear ready to detach. Techniques such as~~ loads are present, sonication or repeated freeze–thawing ~~are~~ is used to release the intracellular virus from the cytoplasm. The lysate may then be clarified using centrifugation to remove cellular debris ~~(for example by use of centrifugation at 600 *g* for 20 minutes~~, with retention of the supernatant~~)~~. ~~A second passage of the virus may be required to produce sufficient virus for a production batch.~~

An aliquot of the virus suspension is titrated to check the virus titre. For LAV, the virus-containing suspension is diluted to attain the dose at which the vaccine candidate will be evaluated or to at least the determined protective dose for approved vaccines and is then mixed with a suitable protectant ~~such as an equal volume of sterile, chilled 5% lactalbumin hydrolysate and 10% sucrose (dissolved in double-~~

~~distilled water or appropriate balanced salt solution),~~ and transferred to individually ~~numbered~~ labelled bottles or bags for storage at low temperatures such as –80°C, or for freeze–drying. A written record of all the procedures followed must be kept for all vaccine batches.

### ~~2.2.2. Requirements for substrates and media~~

~~The specification and source of all ingredients used in the manufacturing procedure should be documented and the freedom of extraneous agents (bacteria, fungi, mycoplasma and viruses) should be tested. The detailed testing procedure is described in Chapter 1.1.9. The use of antibiotics must meet the requirements of the licensing authority.~~

### ~~2.2.3. In-process control~~

~~i) Cells~~

~~Records of the source of the master cell stocks should be maintained. The highest and lowest passage numbers of the cells that can be used for vaccine production must be indicated in the Outline of the Production. The use of a continuous cell line (such as MDBK, etc.) is strongly recommended, unless the virus strain only grows on primary cells. The key advantage of continuous over primary cell lines is that there is less risk of introduction of extraneous agents.~~

~~ii) Serum~~

~~Serum used in the growth or maintenance medium must be free from antibodies to capripoxvirus and free from contamination with pestivirus or other viruses, extraneous bacteria, mycoplasma or fungi.~~

~~iii) Medium~~

~~Media must be sterile before use.~~

~~iv) Virus~~

~~Seed virus and final vaccine must be titrated and pass the minimum release titre set by the manufacturer. For example, the minimum recommended field dose of the South African Neethling strain vaccines (Mathijs *et al.*, 2016) is $\log_{10}$ 3.5 $TCID_{50}$, although the minimum protective dose is $\log_{10}$ 2.0 $TCID_{50}$. Capripoxvirus is highly susceptible to inactivation by sunlight and allowance should be made for loss of activity in the field.~~

~~The recommended field dose of the Romanian sheep pox vaccine for cattle is $\log_{10}$ 2.5 sheep infective doses ($SID_{50}$), and the recommended dose for cattle of the RM65-adapted strain of Romanian sheep pox vaccine is $\log_{10}$ 3 $TCID_{50}$ (Coakley & Capstick, 1961).~~

### 3.2.2. Inactivation process for inactivated LSD vaccines

Unlike LAV, inactivated LSD vaccines contain inactivated antigens in combination with adjuvants to strengthen the induced immune response after administration. The vaccine evaluation process described below needs to show the amount of antigen necessary to elicit a protective immune response. Currently, literature data indicate that an inactivated vaccine originating from an LSDV virus stock with titre $10^4$ cell culture infectious dose$_{50}$ ($CCID_{50}$)/ml before inactivation can be sufficient to induce an efficient immune response to prevent clinical disease, viremia and virus shedding after challenge of young cattle (Wolf *et al.*, 2022)

To monitor the inactivation process and the level of antigen inactivation, samples are taken at regular intervals during inactivation and titrated. Inactivation conditions and the length of initial and repeated exposure should be documented in detail since one or more factors during the process could influence the outcomes. The inactivation kinetics should reach a predefined target e.g. one remaining infectious unit per million doses ($1 \times 10^{-6}$ infectious units/dose) as suggested by APHIS (2013). The confirmatory testing of inactivation is performed on each vaccine lot and represents an important part of the inactivation process monitoring. In addition to all the procedures mentioned above, the inactivation procedure and tests demonstrating that antigen inactivation is complete and consistent must additionally be documented in the Outline of Production.

577 **3.3. Vaccine safety**

578 During the vaccine development process, vaccine safety must be evaluated in the target animal (target animal
579 batch safety test –TABST) to demonstrate the safety of the dose intended for registration. The animals used in
580 the safety testing should be representative (species, age and category [calves, heifers, bulls, cows.]) for all the
581 animals for which the vaccine is intended. Vaccinated and control groups are appropriately acclimatised, housed
582 and managed in line with animal welfare standards. Animal suffering has to be eliminated or reduced and
583 euthanasia is recommended in moribund animals.

584 Essential parameters to be evaluated in safety studies are local and systemic reactions to vaccination, including
585 local reactions at the site of administration, fever, effect on milk production, and induction of a 'Neethling'
586 response. The effect of the vaccine on reproduction needs to be evaluated where applicable.

587 A part of the safety evaluation of LAV and IV can be performed during the efficacy trials (see Section C.3.4
588 *Vaccine efficacy*) by measuring local and systemic responses following vaccination and before challenge.

589 Guidelines for safety evaluation are provided by the European Medicine Agency (EMEA) in VICH GL44: TABST
590 for LAV and IV (EMEA, 2009). Safety aspects of LAV and IV against LSD to be evaluated are:

591 **3.3.1. Overdose test for LAV**

592 Local and systemic responses should be measured following an overdose test whereby 10× the
593 maximum vaccine titre is administered. If the maximum vaccine titre is not specified, 10× the minimum
594 vaccine titre can be applied in multiple injection sites. Ideally, the 10× dose is dissolved in the 1× dose
595 volume of the adjuvants or diluent. Generally, eight animals per group should be used (EMEA, 2009).

596 **3.3.2. One dose and repeat dose test**

597 This aims to test the safety of the vaccine dose applied in the vaccination regime intended for registration.
598 LAV LSD vaccines require one dose per year, while inactivated LSD vaccines require a booster dose in
599 addition to the primary dose. The minimal recommended interval between administrations is 14 days.

600 Generally, eight animals per group should be used unless otherwise justified (EMEA, 2009). For each
601 target species, the most sensitive breed, age and sex proposed on the label should be used.
602 Seronegative animals should be used. In cases where seronegative animals are not reasonably
603 available, alternatives should be justified.

604 **3.3.3. Reversion to virulence tests**

605 Live attenuated vaccines inherently carry the risk of vaccine virus reverting to virulence when repeated
606 passages in a host species could occur due to shedding and transmission from vaccinated animals to
607 contact animals. LAV LSD vaccines should therefore be tested for non-reversion to virulence by means
608 of passage studies. Vaccine virus (MSV, not the finished vaccine) is inoculated in a group of target
609 animals of susceptible age via the natural route of infection or the route that is most likely to result in
610 infection. The vaccine virus is subsequently recovered from tissues or excretions and is used directly to
611 inoculate a further group of animals. After not less than four passages (see chapter 1.1.8), i.e. use of a
612 total of five groups of animals, the re-isolate must be fully characterised, using the same procedures
613 used to characterise the master seed virus.

614 **3.3.4. Environmental consideration**

615 This includes the evaluation of the ability of LAV LSD vaccines to be shed, to spread and to infect contact
616 target and non-target animals, and to persist in the environment.

617 ~~**2.2.4. Final product batch tests**~~

618 ~~i) Sterility/purity~~

619 ~~Vaccine samples must be tested for sterility/purity. *Tests for sterility and freedom from*~~
620 ~~*contamination of biological materials intended for veterinary use* may be found in Chapter 1.1.9.~~

621 ~~ii) Safety and efficacy~~

622 ~~The efficacy and safety studies should be demonstrated using statistically valid vaccination–~~
623 ~~challenge studies using seronegative young LSDV susceptible dairy cattle breeds. The group~~
624 ~~numbers recommended here can be varied if statistically justified. Fifteen cattle are placed in a high~~

625 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.

641 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.

644 iii) Batch potency

645 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. $Log_{10}$ 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 $>\log_{10} 2.5$ is taken as evidence of protection.

657 **3.4. Vaccine efficacy**

658 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 produced at the highest passage level permitted for vaccine production as specified in the Outline of Production.

663 Efficacy (and safety) should be demonstrated in vaccination–challenge studies using representative (by species, age and category) seronegative healthy animals for which the vaccine is intended and which are tested negative for standard viral pathogens.

666 An example of a vaccination-challenge test set-up is outlined here. The group numbers mentioned can be varied if statistically justified. Thirteen animals are placed in a high containment large animal unit and are divided into two groups:

669 - single/repeated dose test group (*n*=8) – animals inoculated with the vaccine dose and route intended for registration (in case of an IV against LSD, a booster dose should follow primary vaccination after minimum 14 days).

672 - control group (*n*=5) – non-vaccinated animals

673 Throughout the *in-vivo* study, all animals are clinically examined and rectal temperatures recorded. Blood, serum and swab samples are regularly collected and subjected to laboratory testing. On day 21 after the vaccination with a LAV or after the booster vaccination for an IV, the animals in both groups are challenged with a known virulent LSDV strain. The challenge virus solution should be of known titre and tested free from extraneous viruses. Experience obtained from previous animal experiments indicates that a dose of challenge virus between $10^{4.0}$ and $10^{6.5}$ $TCID_{50}$ produces clinical disease in about half of the susceptible experimental cattle (Tuppurainen *et al.*, 2021).

The clinical response following challenge is recorded over a period of 14 days. No clinical signs should occur in the vaccinates, other than a local reaction at the site of inoculation. At least 1 animal in the unvaccinated control group should develop the typical clinical signs of LSD. Although a generalised disease with skin nodules may not be seen in all the unvaccinated control animals based on the knowledge that the outcome of a LSDV infection can range from inapparent to severe, at the very least a large local reaction is to be expected.

Clinical and laboratory results will enable assessment of the safety and efficacy of the LSD vaccine candidate and the induced immune responses. Serum samples collected at different time points during the trial can be examined to study seroconversion against selected viral diseases that could have contaminated the vaccine.

## ~~2.3. Requirements for regulatory approval~~

### ~~2.3.1. Safety requirements~~

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

~~The vaccine must be safe to use in all breeds of cattle for which it is intended, including young and pregnant animals. It must also be non-transmissible and remain attenuated after further tissue culture passage.~~

~~Safety tests should be carried out on the final product of each batch as described in Section C.2.2.4.~~

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

~~The selected final vaccine should not revert to virulence during further passages in target animals.~~

~~iii) Environmental consideration~~

~~Attenuated vaccine should not be able to perpetuate autonomously in a cattle population. Strains of LSDV are not a hazard to human health.~~

### ~~2.3.2. Efficacy requirements~~

~~i) For animal production~~

~~The efficacy of the vaccine must be demonstrated in statistically valid vaccination challenge experiments under laboratory conditions. 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 field dose of the vaccine, 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 using intravenous and intradermal inoculation (the challenge virus solution should also be tested and shown to be 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 challenge with LSDV, generalised disease may not be seen in all of the unvaccinated control animals, although there should be a large local reaction.~~

~~Once the potency 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.~~

~~ii) For control and eradication~~

~~Vaccination is the only effective way to control LSD outbreaks in endemic countries and recent experiences of the disease in Eastern Europe and the Balkans suggests this is also true for outbreaks in non-endemic countries. Unfortunately, currently no marker vaccines allowing a DIVA strategy are available, although to a limited extent PCR can be used for certain vaccines.~~

~~The duration of immunity produced by LSDV vaccine strains is currently unknown.~~

#### ~~2.3.3. Stability~~

~~All vaccines are initially given a shelf life of 24 months before expiry. Real-time stability studies are then conducted to confirm the appropriateness of the expiry date. Multiple batches of the vaccine should be re-titrated periodically throughout the shelf-life period to determine the vaccine stability.~~

~~Properly freeze-dried preparations of LSDV vaccine, particularly those that include a protectant, such as sucrose and lactalbumin hydrolysate, are stable for over 25 years when stored at –20°C and for 2–4 years when stored at 4°C. There is evidence that they are stable at higher temperatures, but no long-term controlled experiments have been reported. No preservatives other than a protectant, such as sucrose and lactalbumin hydrolysate, are required for the freeze-dried preparation.~~

### 3.5. Batch/serial tests before release for distribution

Quality tests on MSV and safety and efficacy tests on vaccine candidates are performed during the evaluation process for new LSD vaccines. Once vaccines are approved to be used in the field, it remains important to verify the quality of each vaccine batch produced. An independent batch quality control assessment may be warranted or requested by national or international regulatory authorities.

#### 3.5.1. Purity test

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.

Besides the contaminants mentioned above, manufacturers should demonstrate implemented measures to minimise the risk of TSE contamination in ingredients of animal origin such as:

- all ingredients of animal origin in production facilities are from countries recognised as having the lowest possible risk of bovine spongiform encephalopathy
- tissues or other substances used are themselves recognised as being of low or nil risk of containing TSE agents

#### 3.5.2. Identity tests

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).

#### 3.5.3. Potency tests

Standard requirements for potency tests can be found in CFR Title 9 part 113, in the European Pharmacopoeia, and in this Terrestrial Manual.

##### 3.5.3.1. Live vaccines

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 $10^3$ and $10^4$ infectious units/dose (Tuppurainen *et al*., 2021).

##### 3.5.3.2. Inactivated LSD vaccines

For inactivated LSD vaccines, potency tests are performed using vaccination–challenge efficacy studies in animal hosts (see Section C.3.4. *Vaccine efficacy*).

774 **3.5.4. Safety/efficacy**

775 Safety and efficacy testing is undertaken during the evaluation process of the vaccine candidate, and
776 also needs to be performed on a number of vaccine batches until robust data are generated in line with
777 international and national regulations. Afterwards, when using a seed lot system in combination with
778 strict implementation of GMP standards and depending on local regulations, TABST could be waived as
779 described in VICH50 and VICH55, providing the titer has been ascertained using potency testing.
780 Batches or serials are considered satisfactory if local and systemic reactions to vaccination are in line
781 with those described in the dossier of the vaccine candidate and product literature.

782 **3.5.4.1. Field safety/efficacy tests**

783 Field testing of two or more batches should be performed on all animal categories for which the
784 product is indicated before release of the product for general use (see chapter 1.1.8). The aim of
785 these studies is to demonstrate the safety and efficacy of the product under normal field conditions
786 of animal care and use in different geographical locations where different factors may influence
787 product performance. A protocol for safety/efficacy testing in the field has to be developed with
788 defined observation and recording procedures. However, it is generally more difficult to obtain
789 statistically significant data to demonstrate efficacy under field conditions. Even when properly
790 designed, field efficacy studies may be inconclusive due to uncontrollable outside influences.

791 **3.5.4.2. Duration of Immunity**

792 The duration of immunity (DOI) following vaccination should be demonstrated via challenge or the
793 use of a validated serology test. Efficacy testing at the end of the claimed period of protection should
794 be conducted in each species for which the vaccine is indicated or the manufacturer should indicate
795 that the DOI for that species is not known. Likewise, the manufacturer should demonstrate the
796 effectiveness of the recommended booster regime in line with these guidelines, usually by
797 measuring the magnitude and kinetics of the serological response observed.

798 ## ~~3. Vaccines based on biotechnology~~

799 ~~A new generation of capripox vaccines is being developed that uses the LSDV as a vector for the expression and delivery~~
800 ~~of immuno-protective proteins of other ruminant pathogens with the potential for providing dual protection (Boshra *et al.*,~~
801 ~~2013; Wallace & Viljoen, 2005), as well as targeting putative immunomodulatory genes for inducing improved immune~~
802 ~~responses (Kara *et al.*, 2018).~~

803 ## 4. Post-market studies

804 ### 4.1. Stability

805 Stability testing shall be carried out as specified in Annex II of Regulation (EU) 2019/6 and in the Ph. Eur. 0062:
806 Vaccines for veterinary use, on not fewer than three representative batches providing this mimics the full-scale
807 production described in the application. At the end of shelf-life, sterility has to be re-evaluated using sterility
808 testing or by showing container closure integrity. Multiple batches of the vaccine should be re-titrated periodically
809 throughout the shelf-life period to determine the vaccine stability.

810 ### 4.2. Post-marketing surveillance

811 After release of a vaccine, its performance under field conditions should continue to be monitored by competent
812 authorities and by the manufacturer itself. Not all listed adverse effects may show up in the clinical trials
813 performed to assess safety and efficacy of the vaccine candidate due to the limited number of animals used.
814 Post-marketing surveillance studies can also provide information on vaccine efficacy when used in normal
815 practice and husbandry conditions, on duration of induced immunity, on ecotoxicity, etc.

816 First, a reliable reporting system should be in place to collect consumer complaints and notifications of adverse
817 reactions. Secondly, post-marketing surveillance should be established to investigate whether the reported
818 observations are related to the use of the product and to identify, at the earliest stage, any serious problem that
819 may be encountered from its use and that may affect its future uptake. Vaccinovigilance should be an on-going
820 and integral part of all regulatory programmes for LSD vaccines, especially for live vaccines.

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971 * *

972 **NB:** There are WOAH Reference Laboratories for lumpy skin disease (please consult the WOAH Web site:
973 https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
974 Please contact WOAH Reference Laboratories for any further information on
975 diagnostic tests, reagents and vaccines for lumpy skin disease

976 **NB:** FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2021.

**MEETING OF THE BIOLOGICAL STANDARDS COMMISSION**

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# CHAPTER 3.6.9.

# EQUINE RHINOPNEUMONITIS (INFECTION WITH VARICELLOVIRUS EQUIDALPHA1 ~~EQUID HERPESVIRUS-1 AND -4~~)

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## 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 WOAH 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~~s~~ 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~~.*

***Serological tests:** As most horses possess some level of antibody to EHV-1/4, the demonstration of specific antibody in the serum collected from a single blood sample is ~~therefore~~ not confirmation of ~~a positive diagnosis of~~ recent infection. Paired~~,~~ (acute and convalescent) sera from animals suspected of being infected with EHV-1 or EHV-4 should be tested for a four-fold or greater rise in virus-specific antibody titre by either virus neutralisation (VN) or complement fixation (CF)* tests. *Neither of these assays is type-specific but both have proven useful for diagnostic purposes especially since the CF antibody response to recent infection is relatively short-lived. Limited use has also been made of a type-specific enzyme-linked immunosorbent assay ~~(Crabb et al., 1995; Hartley et al., 2005)~~.*

***Requirements for vaccines:** Both live attenuated and inactivated viral vaccines are available for use in assisting in the control of EHV-1/4. Vaccination is helpful in reducing the severity of respiratory infection in young horses and the incidence of abortion in mares; however current vaccines are not licenced to protect against neurological disease. Vaccination should not be considered a substitute for sound management practices known to reduce the risk of infection. Revaccination at frequent intervals is recommended in the case of each of the products, as the duration of vaccine-induced immunity is relatively short.*

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

# A. INTRODUCTION

Equine rhinopneumonitis (ER) is a historically derived term that describes a constellation of several disease entities of horses that may include respiratory disease, abortion, neonatal foal pneumonitis, or myeloencephalopathy (~~Allen & Bryans, 1986;~~ Allen *et al*., 1999~~; Bryans & Allen, 1988; Crabb & Studdert, 1995~~). The disease ~~has been~~ is recognised ~~for over 60 years~~ as a threat to the international horse industry, and is caused by either of two members of the *Herpesviridae* family, formerly known as equid alphaherpesvirus-1 and -4 (EHV-1 and EHV-4). The viruses are now classified as Varicellovirus equidalpha1 and Varicellovirus equidalpha4. For the purposes of the chapter, the acronyms EHV-1 and EHV-4 will continue to be used. EHV-1 and EHV-4 are closely related alphaherpesviruses of horses with nucleotide sequence identity within individual homologous genes ranging from 55% to 84%, and amino acid sequence identity from 55% to 96% (Telford *et al*., ~~1992;~~ 1998). ~~The two herpesviruses~~ With the exception of EHV-1 in Iceland (Thorsteinsdóttir *et al*., 2021), the two herpesviruses are considered endemic ~~enzootic~~ in all countries in which large populations of horses are maintained as part of the cultural tradition or agricultural economy. There is no recorded evidence that the two herpesviruses of ER pose any health risks to humans working with the agents. Infection with EHV-1 is listed by WOAH and is therefore the focus of this chapter.

Viral transmission to cohort animals occurs by inhalation of aerosols of virus-laden respiratory secretions. Morbidity tends to be highest in young horses sharing the same air space. Aborted tissues and placental fluids from infected mares can contain extremely high levels of live virus and represent a major source of infection. Extensive use of vaccines has not eliminated EHV-1 infections, and the world-wide ~~annual~~ financial impact from this ~~these~~ equine pathogen~~s~~ is ~~immense~~ considerable.

In horses under 3 years of age, clinical ER usually takes the form of an acute, febrile respiratory illness that spreads rapidly through the group of animals. The virus~~es~~ infects and multiplies ~~multiply~~ in epithelial cells of the respiratory mucosa. Signs of infection become apparent 2–8 days after exposure to virus, and are characterised by fever, inappetence, depression, and nasal discharge. The severity of respiratory disease varies with the age of the horse and the level of immunity resulting from previous vaccination or natural exposure. Bi-phasic fever, viraemia and complications are more likely with EHV-1 than EHV-4. Subclinical infections with EHV-1/4 are common, even in young animals. Although mortality from uncomplicated ER is rare and complete recovery within 1–2 weeks is the normal outcome, respiratory infection is a frequent and significant cause of interrupted schedules among horses assembled for training, racing, or other equestrian events. Fully protective immunity resulting from infection is of short duration, and convalescent animals are susceptible to reinfection by EHV-1/4 after several months. Although reinfections ~~by the two herpesviruses~~ cause less severe or clinically inapparent respiratory disease, the risks of subsequent abortion or neurological disease remain. Like other herpesviruses, EHV-1/4 causes long-lasting latent infections and latently infected horses represent a potential infection risk for other horses. Virus can be reactivated as a result of stress ~~or pregnancy~~. The greatest clinical threats to individual breeding, racing, or pleasure horse operations posed by ER are the potential abortigenic and neurological *sequelae* of EHV-1 respiratory infection. ER abortions occur annually in horse populations worldwide and may be sporadic or multiple. Foals infected *in utero* may be born alive and die within a few days of birth. EHV-1 neurological disease is less common than abortions

but has been recorded all over the world with associated fatalities. Outbreaks result in movement restrictions and, sometimes, cancellation of equestrian events (Couroucé *et al*., 2023; FEI, 2021).

~~Neurological disease, also known as equine herpesvirus myeloencephalopathy, remains an infrequent but serious complication of EHV-1 infection. A single mutation in the DNA polymerase gene (ORF30) has been associated with increased risk of neurological disease, however strains without this change can also cause paralysis (Goodman *et al*., 2007; Nugent *et al*., 2006). Strain typing techniques have been employed to identify viruses carrying the neuropathic marker, and it can be helpful to be aware of an increased risk of neurological complications. However, for practical purposes strain typing is not relevant for agent identification, or international trade. Strain typing may be beneficial for implementation of biosecurity measures in the management of outbreaks of equine herpesvirus myeloencephalopathy.~~

Strain typing has been shown to be not reliable for predicting the clinical outcome of EHV-1 infection but can be useful in epidemiological investigations (Garvey *et al*., 2019; Nugent *et al*., 2006; Sutton *et al*., 2019).

EU: Strain typing has been shown to be unreliable ~~not reliable~~ for predicting the clinical outcome of EHV-1 infection but can be useful in epidemiological investigations (Garvey *et al*., 2019; Nugent *et al*., 2006; Sutton *et al*., 2019)."

# B. DIAGNOSTIC TECHNIQUES

~~Both~~ EHV-1 ~~and EHV-4~~ is transmitted by the respiratory route and has ~~have~~ the potential to be highly contagious~~, viruses~~ particularly where large numbers of horses are housed in the same air space. EHV1 ~~and the former~~ can cause explosive outbreaks of abortion or neurological disease. Rapid diagnostic methods are therefore essential ~~useful~~ for managing the disease. Real-time polymerase chain reaction (PCR) assays are ~~widely~~ routinely used by diagnostic laboratories worldwide and are both rapid and sensitive. Real-time PCR assays ~~that allow simultaneous testing for EHV-1 and EHV-4~~ have been developed for both detection of EHV-1 and quantification of viral load ~~have been developed.~~ and have replaced virus isolation ~~has been replaced by real-time PCR~~ as the frontline diagnostic test in the majority of laboratories. ~~but~~ Virus isolation can ~~also~~ still be useful~~, particularly for the detection of viraemia. This is also true of for~~ in cases of EHV-1-associated abortions and neonatal foal deaths, when 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 extremely useful~~ for rapid diagnosis of EHV-induced abortion from fresh or embedded tissue ~~and are relatively straightforward. Several other techniques based on enzyme-linked immunosorbent assay (ELISA) or nucleic acid hybridisation probes have also been described, however their use is often restricted to specialised laboratories and they are not included here.~~ Virus neutralisation (VN) and complement fixation test (CFT) are the most frequently used serological tests, and seroconversion in paired samples is considered indicative of exposure to virus by natural infection or by vaccination.

***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 | – | +++ | – | +++ | – | – |
| Direct immunofluorescence | – | – | – | ++ | – | – |
| Detection of immune response | | | | | | |
| VN | ++ | ++ | – ~~+~~ | ++ ~~+~~ | +++ | +++ |
| ELISA | + | – ++ | – ~~+~~ | ++ | ++~~+~~ | ++ |

| 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)] |
| CFT | – | – ++ | – | +++ | – | – +++ |

131 Key: +++ = recommended for this purpose; ++ recommended but has limitations;
132 + = suitable in very limited circumstances; – = not appropriate for this purpose.
133 PCR = polymerase chain reaction; VN = virus neutralisation;
134 ELISA = enzyme-linked immunosorbent assay; CFT = complement fixation test.
135 [(a)]See Appendix 1 of this chapter for justification table for the scores giving to the tests for this purpose.
136 [(b)]See Appendix 2 of this chapter for justification table for the scores giving to the tests for this purpose.
137 [(c)]No eradication policies exist for equine rhinopneumonitis.
138 [(d)]See Appendix 4 of this chapter for justification table for the scores giving to the tests for this purpose.
139 [(e)]See Appendix 5 of this chapter for justification table for the scores giving to the tests for this purpose.
140 [(f)]See Appendix 6 of this chapter for justification table for the scores giving to the tests for this purpose.
141 [(g)]A combination of agent identification methods applied on the same clinical sample is recommended.

## 142 1. Identification Detection of the agent

### 143 1.1. Collection and preparation of specimens

144 *Nasal/nasopharyngeal swabs:* swab extract can be used for DNA extraction and subsequent virus
145 detection by PCR using one of a variety of published techniques or commercially available kits (see
146 below). Virus isolation can also be attempted from the swab extracts. To increase the chances of isolating
147 live virus, swabs are best obtained from horses during the very early, febrile stages acute stage of the
148 respiratory disease, and are collected via the nares by sampling the area with a swab of an appropriate
149 size and length for horses. After collection, the swab should be removed and transported immediately to
150 the virology laboratory in 3 ml of cold (not frozen) virus transport medium (e.g. phosphate buffered saline
151 [PBS] or serum-free MEM [minimal essential medium] with antibiotics). Virus infectivity can be prolonged
152 by the addition of bovine serum albumin, fetal calf serum or gelatine to 0.1% (w/v).

153 *Tissue samples:* total DNA can be extracted using a number of commercially available kits and used in
154 PCR to detect viral DNA (described below in Section B.1.2.1). Virus isolation from placenta and fetal
155 tissues from suspect cases of EHV-1 abortion is most successful when performed on aseptically
156 collected samples of placenta, liver, lung, thymus, adrenal glands and spleen. Virus may be isolated from
157 post-mortem cases of EHV-1 neurological disease by culture of samples of brain and spinal cord but
158 such attempts to isolate virus are often unsuccessful; however, they these samples may be useful for
159 PCR testing and pathological examination. Tissue samples should be transported to the laboratory and
160 held at 4°C until inoculated into tissue culture. Samples that cannot be processed within a few hours
161 should be stored at –70°C.

162 *Blood:* for virus detection by PCR or isolation from blood leukocytes, collect a 10–20 ml sample of blood,
163 using an aseptic technique in citrate, heparin or EDTA [ethylene diamine tetra-acetic acid] anticoagulant.
164 EDTA is the preferred anticoagulant for PCR testing in some laboratories as heparin may inhibit DNA
165 polymerase. The samples should be transported without delay to the laboratory on ice, but not frozen.

166 *Cerebrospinal fluid:* the detection of EHV-1 DNA in cerebrospinal fluid has been reported in cases of
167 neurological disease.

### 168 1.2. Virus detection by polymerase chain reaction

169 PCR has become the primary diagnostic method for the detection of EHV-1 and EHV-4 in clinical
170 specimens, paraffin-embedded archival tissue, or inoculated cell cultures (Borchers & Slater, 1993;
171 Lawrence *et al.*, 1994; O'Keefe *et al.*, 1994; Varrasso *et al.*, 2001). A variety of type-specific PCR primers
172 have been designed to distinguish between the presence of EHV-1 and EHV-4. The correlation between
173 PCR and virus isolation techniques for diagnosis of EHV-1 or EHV-4 is high (Varrasso *et al.*, 2001).
174 Diagnosis by PCR is rapid, sensitive, and does not depend on the presence of infectious virus in the
175 clinical sample. For diagnosis of active infection by EHV, PCR methods are routinely used to detect EHV-
176 1 DNA in nasopharyngeal swabs and tissue samples most reliable with tissue samples from aborted
177 fetuses and placental tissue or from nasopharyngeal swabs of foals and yearlings. They are particularly
178 useful in explosive outbreaks of abortion, respiratory or neurological disease, in which a rapid

identification and monitoring of the virus spread is critical for guiding management strategies, including movement restrictions. PCR examination of spinal cord and brain tissue, as well as peripheral blood mononuclear cells (PBMC), are important in seeking a diagnosis on a horse with neurological signs (Pronost *et al*., 2012).

~~Several PCR assays have been published. A nested PCR procedure can be used to distinguish between EHV-1 and EHV-4. A sensitive protocol suitable for clinical or pathological specimens (nasal secretions, blood leukocytes, brain and spinal cord, fetal tissues, etc.) has been described by Borchers & Slater (1993). However, nested PCR methods have a high risk of laboratory cross-contamination, and sensitive rapid one-step PCR tests to detect EHV-1 and EHV-4 (e.g. Lawrence *et al*., 1994) are preferred. The WOAH Reference Laboratories use quantitative real-time PCR assays such as those targeting heterologous sequences of major glycoprotein genes to distinguish between EHV-1 and EHV-4. A multiplex real-time PCR targeting glycoprotein B gene of EHV-1 and EHV-4 was described by Diallo *et al*. (2007). PCR protocols have been developed that can differentiate between EHV-1 strains carrying the ORF30 neuropathogenic marker, using both restriction enzyme digestion of PCR products (Fritsche & Borchers, 2011) or by quantitative real-time PCR (Allen *et al*, 2007, Smith *et al*., 2012). Methods have also been developed to type strains for epidemiological purposes, based on the ORF68 gene (Nugent *et al*., 2006). The WOAH Reference Laboratories employ in-house methods for strain typing, however these protocols have not yet been validated between different laboratories at an international level.~~

Real-time (or quantitative) PCR has become the method of choice for ~~many~~ the majority of diagnostic ~~tests~~ laboratories and provides rapid and sensitive detection of viral DNA. Equine post-mortem tissues from newborn and adult animals or equine fetal tissue from abortions (tissues containing lung, liver, spleen, thymus, adrenal gland and placental tissues) can be used. For respiratory samples, equine nasopharyngeal swabs or deep nasal swabs (submitted in a suitable viral transport medium), buffy coat, tracheal wash (TW) or broncho-alveolar lavage (BAL) are all suitable. DNA should be extracted using an appropriate kit or robotic system.

There is no internationally standardised real-time PCR method for EHV-1 but Table 2 summarises the primer and probe sequences for some of the most widely used assays. Type-specific PCR primers have been designed to distinguish between the presence of EHV-1 and EHV-4. The optimised thermocycler times and temperatures are documented in the publications cited.

***Table 2.*** *Primer and probe sequences for EHV1/4 detection by real-time PCR*

| Primer | Primer sequence (5' to 3') | Target | Reference |
|---|---|---|---|
| ~~Forward~~ | ~~CAT-GTC-AAC-GCA-CTC-CCA~~ | ~~EHV-1 gB~~ | ~~Diallo *et al*., 2006~~ |
| ~~Reverse~~ | ~~GGG-TCG-GGC-GTT-TCT-GT~~ | | |
| ~~Probe~~ | ~~FAM-CCC-TAC-GCT-GCT-CC-MGB-NFQ~~ | | |
| Forward | CAT-ACG-TCC-CTG-TCC-GAC-AGA-T | EHV-1 gB | Hussey *et al*., 2006 |
| Reverse | GGTACTCGGCCTTTGACGAA | | |
| Probe | FAM-TGA-GAC-CGA-AGA-TCT-CCT-CCA-CCG-A-BHQ1 | | |
| Forward | TAT-ACT-CGC-TGA-GGA-TGG-AGA-CTT-T | EHV-1 gB | Pusterla *et al*., 2009 |
| Reverse | TTG-GGG-CAA-GTT-CTA-GGT-GGT-T | | |
| Probe | 6FAM-ACA-CCT-GCC-CAC-CGC-CTA-CCG | | |
| Forward | GCG-GGC-TCT-GAC-AAC-ACA-A | EHV-1 gC | ISO 17025 accredited for the detection of EHV-1 at WOAH Reference Laboratory |
| Reverse | TTG-TGG-TTT-CAT-GGG-AGT-GTG-TA | | |
| Probe | FAM-TAA-CGC-AAA-CGG-TAC-AGA-A-BHQ1 | | |

~~*This multiplex real-time PCR test has been validated to ISO 17025 and is designed for use in a 96-well format. This can be readily combined with automatic nucleic acid extraction methods. Discrimination between EHV-1 and EHV-4 is carried out by the incorporation of type-specific dual labelled probes based on methods published by Hussey *et al*. (2006) and Lawrence *et al*. (1994). To establish such a real-time PCR assay for diagnostic purposes, validation against blinded samples is required. Sensitivity and specificity for the assay should be determined against each target. Support for development of assays and appropriate sample panels can be obtained from the WOAH Reference Laboratories.~~ Reference material and sample panels for real-time PCR can be obtained from the WOAH Reference Laboratories.

- Point of care (POC) molecular tests

Loop-mediated isothermal amplification (LAMP) assays for the detection of EHV-1 have been described (Nemoto *et al.*, 2011). An evaluation of a hydrolysis probe-based insulated isothermal PCR (iiPCR) assay for the detection of EHV-1 showed it to have a high sensitivity and specificity compared with real-time PCR (Balasuriya *et al.*, 2017). However further validation of POC tests in the field is required.

- Molecular characterisation

Allelic discrimination real-time PCR assays identifying a single nucleotide polymorphism that was originally suggested to distinguish between neuropathogenic and non-neuropathogenic EHV-1 strains have been developed (Smith *et al.*, 2012). However, investigations in many countries worldwide demonstrated that the nucleotide substitution was not a reliable predictor of enhanced neuropathogenicity. Multilocus typing and whole genome sequencing are useful for molecular epidemiological studies (Garvey *et al.*, 2019; Nugent *et al.*, 2006; Sutton *et al.*, 2019).

### 1.3. Virus isolation

Virus isolation is no longer a routine test used for EHV-1 detection in the majority of diagnostic laboratories but is more often conducted for surveillance and research purposes. A number of cell types may be used for isolation of EHV-1 (e.g. rabbit kidney [RK-13 (AATC–CCL37)], baby hamster kidney [BHK-21], Madin–Darby bovine kidney [MDBK], pig kidney [PK-15], etc.). RK13 cells are commonly used for this purpose. ~~For efficient primary isolation of EHV-4 from horses with respiratory disease, equine-derived cell cultures must be used. Both EHV-1 and EHV-4 may be isolated from nasopharyngeal samples using primary equine fetal kidney cells or equine fibroblasts derived from dermal (E-Derm) or lung tissue. EHV-1 can be isolated on other cell types, as will be discussed later. The nasopharyngeal swab and its accompanying transport medium are transferred into the barrel of a sterile 10 ml syringe. Using the syringe plunger, the fluid is squeezed from the swab into a sterile tube. A portion of the expressed fluid can be filtered through a sterile, 0.45 µm membrane syringe filter unit into a second sterile tube if heavy bacterial contamination is expected, but this may also lower virus titre. Recently prepared cell monolayers in tissue culture flasks are inoculated with the filtered, as well as the unfiltered, nasopharyngeal swab extract. Cell monolayers in multiwell plates incubated in a 5% $CO_2$ environment may also be used. Virus is allowed to attach by incubating the inoculated monolayers at 37°C. Monolayers of uninoculated control cells should be incubated in parallel.~~

~~At~~ Recently prepared cell monolayers in tissue culture flasks or plates are inoculated with nasopharyngeal swab extract or homogenised tissue: approximately 10% (w/v) pooled tissue homogenates of liver, lung, thymus, adrenal gland and spleen (from aborted fetuses/neonatal foals) or of brain and spinal cord (from cases of neurological disease). Virus is allowed to attach by incubating the ~~end of the attachment period,~~ inoculated monolayers at 37°C for 1 hour after which the inocula are removed and the monolayers are rinsed twice with PBS ~~to remove virus-neutralising antibody that may~~ or maintenance medium. Monolayers of uninoculated control cells should be ~~present in the nasopharyngeal secretions~~ incubated in parallel. After addition of supplemented maintenance medium (MEM containing 2% fetal calf serum [FCS] and twice the standard concentrations of antibiotics/antifungals [penicillin, streptomycin, gentamicin, and amphotericin B]), the flasks are incubated at 37°C in a 5% $CO_2$ environment.

The use of a positive control ~~virus samples~~ of relatively low titre may be used to validate the isolation procedure ~~carries the risk that this may lead~~ but should be processed separately to ~~eventual~~ avoid contamination of diagnostic specimens. ~~This risk can be minimised by using routine precautions and good laboratory technique, including the use of biosafety cabinets, inoculating positive controls after the diagnostic specimens, decontaminating the surfaces in the hood while the inoculum is adsorbing and using a positive control of relatively low titre.~~ Inoculated flasks should be inspected daily by microscopy for the appearance of characteristic herpesvirus cytopathic effect (CPE) (focal rounding, increase in refractility, and detachment of cells). Cultures exhibiting no evidence of viral CPE after 1 week of incubation should be blind-passaged into freshly prepared monolayers of cells, using small aliquots of both media and cells as the inoculum. Further blind passage is usually not productive.

~~It can be useful to inoculate samples into both non-equine and equine cells in parallel to distinguish between EHV-1 and EHV-4, since EHV-4 can cause sporadic cases of abortion. Around 10% (w/v) pooled tissue homogenates of liver, lung, thymus, and spleen (from aborted fetuses) or of central nervous system tissue (from cases of neurological disease) are used for virus isolation. These are prepared by first mincing small samples of tissue into 1 mm cubes in a sterile Petri dish with dissecting scissors, followed by macerating the tissue cubes further in serum-free culture medium with antibiotics using a homogeniser or mechanical tissue grinder. After centrifugation at 1200* **g** *for 10 minutes, the supernatant~~

~~is removed and 0.5 ml is inoculated into duplicate cell monolayers in tissue culture flasks. Following incubation of the inoculated cells at 37°C for 1.5–2 hours, the inocula are removed and the monolayers are rinsed twice with PBS or maintenance medium. After addition of 5 ml of supplemented maintenance medium, the flasks are incubated at 37°C for up to 1 week or until viral CPE is observed. Cultures exhibiting no evidence of viral CPE after 1 week of incubation should be passaged a second time into freshly prepared monolayers of cells, using small aliquots of both media and cells as the inoculum.~~

Blood samples: EHV-1 ~~and, infrequently, EHV-4~~ can be isolated from PBMC. Buffy coats may be prepared from unclotted (heparinised) blood by centrifugation at ~~600~~ 525 ***g*** for ~~15~~ 5 minutes~~, and~~. The buffy coat is taken after the plasma has been carefully removed~~. The buffy coat is then layered onto a PBMC separating solution (Ficoll; density 1077 g/ml, commercially available) and centrifuged at 400 **g** for 20 minutes. The PBMC interface (without most granulocytes) is~~ and washed ~~twice in PBS (300 **g** for 10 minutes) and resuspended in 1 ml~~ three times in 3 ml MEM containing 2% FCS~~. As a quicker alternative method, PBMC may be collected by centrifugation directly from plasma.~~ (525 ***g*** for 5 minutes). Following the third wash, the buffy coat is harvested and resuspended in 2.5 ml MEM containing 2% FCS. An aliquot of the rinsed cell suspension ~~is added to each of the duplicate monolayers of equine fibroblast, equine fetal or RK-13 cell monolayers in 25 cm² flasks containing 8–10 ml freshly added maintenance medium. The flasks~~ can be used for DNA extraction. For virus isolation, the resuspended cells (1 ml) are co-cultivated with freshly prepared primary equine lung or RK-13 cell suspensions (5 ml) in 25 cm$^2$ flasks. Confluent cell monolayers are not used. The flasks are incubated at 37°C in a 5% $CO_2$ environment for 3 days or until the cells have reached 90% confluence. The monolayers are then rinsed three times with 1 × PBS and supplemented with 5 ml MEM containing 2% FCS. They are incubated ~~at 37°C for 7 days; either with or without removal of the inoculum. If PBMCs are not removed prior to incubation, CPE may be difficult to detect in the presence of the massive inoculum of leukocytes: each flask of cells is freeze–thawed after 7~~ for a further 4 days ~~of incubation and the contents centrifuged at 300 **g** for 10 minutes. Finally, 0.5 ml of the cell-free, culture medium supernatant is transferred to freshly made cell monolayers that are just subconfluent. These are incubated~~ and observed daily for viral CPE ~~for at least 5–6 days. Again, samples~~. Samples exhibiting no evidence of viral CPE after 1 week of incubation should be passaged a second time before discarding as negative.

Virus identity may be confirmed by PCR or by immunofluorescence with specific antisera. Virus isolates from positive cultures should be submitted to a WOAH Reference Laboratory for strain characterisation and to maintain a geographically diverse archive. ~~Further strain characterisation for surveillance purposes or detection of the neurological marker can be provided at some laboratories.~~

### 1.4. Virus detection by direct immunofluorescence

Direct immunofluorescent detection of EHV-1 antigens in samples of post-mortem tissues collected from aborted equine fetuses and the placenta provides a rapid preliminary diagnosis of herpesvirus abortion (Gunn, 1992). The diagnostic reliability of this technique approaches that of virus isolation attempts from the same tissues.

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

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

### 1.5. Virus detection by immunoperoxidase staining

Immunohistochemical (IH) staining methods, such as immunoperoxidase, have been developed for detecting EHV-1 antigen in fixed tissues of aborted equine fetuses, placental tissues or neurologically affected horses (Schultheiss *et al*., 1993; Whitwell *et al*., 1992). Such techniques can be used as an alternative to immunofluorescence described above and can also be readily applied to archival frozen or fixed tissue samples. Immunohistochemical staining for EHV-1 is particularly useful for the simultaneous evaluation of morphological lesions and the identification of the virus. Immunoperoxidase staining for EHV-1/4 may also be carried out on infected cell monolayers ~~(van Maanen *et al*., 2000)~~. Adequate controls must be included with each immunoperoxidase test run for evaluation of both the method

specificity and antibody specificity. ~~In one WOAH Reference Laboratory, this~~ ~~method is used routinely for frozen or fixed tissue, using~~ If non-specific ~~rabbit~~ polyclonal sera is used ~~raised against EHV-1. This staining method is not type-specific and therefore~~ the staining method needs to be combined with virus isolation or PCR to discriminate between EHV-1 and EHV-4~~, however it provides a useful method for rapid diagnosis of EHV-induced abortion~~.

### 1.6. Histopathology

Histopathological examination of sections of fixed placenta and lung, liver, spleen, adrenal gland and thymus from aborted fetuses and brain and spinal cord from neurologically affected horses should be carried out. In aborted fetuses, eosinophilic intranuclear inclusion bodies present within bronchiolar epithelium or in cells at the periphery of areas of hepatic necrosis are consistent with a diagnosis of herpesvirus infection. The characteristic microscopic lesion associated with EHV-1 neuropathy is a degenerative thrombotic vasculitis of small blood vessels in the brain or spinal cord (perivascular cuffing and infiltration by inflammatory cells, endothelial proliferation and necrosis, and thrombus formation).

## 2. Serological tests

EHV-1 and EHV-4 are endemic in most parts of the world and seroprevalence is high, however serological testing of paired sera can be useful for diagnosis of ER in horses. A positive diagnosis is based on the demonstration of significant increases (four-fold or greater) in antibody titres in paired sera taken during the acute and convalescent stages of the disease. The results of tests performed on sera from a single collection date are, in most cases, impossible to interpret with any degree of confidence. The initial (acute phase) serum sample should be taken as soon as possible after the onset of clinical signs, and the second (convalescent phase) serum sample should be taken 2–4 weeks later.

'Acute phase' sera from mares after abortion or from horses with EHV-1 neurological disease may already contain maximal titres of EHV-1 antibody, with no increase in titres detectable in sera collected at later dates. In such cases, serological testing of paired serum samples from clinically unaffected cohort members of the herd may prove useful for retrospective diagnosis of ER within the herd.

~~Finally, the serological detection of antibodies to EHV-1 in heart or umbilical cord blood or other fluids of equine fetuses can be of diagnostic value in cases of abortion especially when the fetus is virologically negative. The EHV 1/4 nucleic acid may be identified from these tissues by PCR.~~

Serum antibody levels to EHV-1/4 may be determined by virus neutralisation (VN) (Thomson *et al*., 1976), complement fixation tests (CFT) (Thomson *et al*., 1976) or enzyme-linked immunosorbent assay (ELISA) (Crabb & Studdert, 1995). ~~There are no internationally recognised reagents or standardised techniques for performing any of the serological tests for detection of EHV-1/4 antibody; titre determinations on the same serum may differ from one laboratory to another. Furthermore,~~ The CF and VN tests detect antibodies that are cross-reactive between EHV-1 and EHV-4. Nonetheless, the demonstration of a four-fold or greater rise in antibody titre to EHV-1 or EHV-4 during the course of a clinical illness provides serological confirmation of recent infection with one of the viruses. Commercial ELISAs that distinguish EHV-1 and EHV-4 antibodies are available but less widely used than the CF and VN tests. ~~Unlike other alphaherpesviruses, DIVA ELISAs, which have been very useful in eradication programmes for bovine rhinotracheitis and pseudorabies (Aujeszky's disease), have not been developed for EHV-1/4.~~ An ELISA using a synthetic peptide for glycoprotein E as an antigen (Andoh *et al.*, 2013) is used as DIVA[1] for horses vaccinated with a modified live EHV-1 vaccine licensed in Japan, that lacks the glycoprotein E gene.

The ~~microneutralisation test is a~~ VN and the CF tests are widely used and sensitive serological assays for detecting EHV-1/4 antibody and will thus be described here.

### 2.1. Virus neutralisation test

This test is most commonly performed in flat-bottom 96-well microtitre plates (tissue culture grade) using a constant dose of virus and doubling dilutions of equine test sera. At least ~~two~~ three replicate wells for each serum dilution are required. Heat-inactivated maintenance medium with a concentration of 2% FCS (HIMM) ~~Serum-free MEM~~ is used throughout as a diluent. Virus stocks of known titre are diluted just before use to contain 100 $TCID_{50}$ (50% tissue culture infective dose) in 25 µl. Monolayers of ~~E-Derm or~~ RK-13 cells are prepared ~~monodispersed with EDTA/trypsin and resuspended at a concentration of 5 × $10^5$/ml~~. ~~Note that RK-13 cells can be used with EHV-1 but do not show CPE with EHV-4.~~ Antibody positive and negative control equine sera and controls for cell viability, virus infectivity, and test serum cytotoxicity, must be included in each assay. End-point VN titres of antibody are calculated by determining the

[1] DIVA: detection of infection in vaccinated animals

reciprocal of the highest serum dilution that protects ≥75% ~~100%~~ of the cell monolayer from virus destruction in ~~both of~~ the replicate wells.

Serum toxicity may be encountered in samples from horses repeatedly vaccinated with a commercial vaccine prepared from EHV-1 grown up in RK-13 cells. This can give rise to difficulties in interpretation of test reactions at lower serum dilutions. The problem can be overcome using E-Derm or other non-rabbit kidney derived cell line.

### 2.1.1. Test procedure

A suitable test procedure is as follows:

i) Prepare semi-confluent monolayers in tissue culture microtitre plates.

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

iii) Add 40 ~~25~~ µl of HIMM ~~serum-free MEM~~ to all wells of the microtitre assay plates.

iv) For test sample titration, pipette ~~25~~ 40 µl of each test serum into ~~duplicate~~ triplicate wells of ~~both rows A and B of~~ the plate. The first two rows serve as the dilution of the test serum and the third row serves as the serum toxicity control ~~and the second row as the first dilution of the test~~. Make doubling dilutions of each serum ~~starting with row B and proceeding to the bottom of the plate~~ by sequential mixing and transfer of ~~25~~ 40 µl to each subsequent row of wells. ~~Six sera can be assayed in each plate.~~ Add 40µl of HIMM to the serum control rows.

v) Add 40 ~~25~~ µl of the appropriately diluted EHV-1 ~~or EHV-4~~ virus stock to ~~each~~ all wells (100 $TCID_{50}$/well) of the test plate except those of ~~row A, which are~~ the serum controls ~~wells~~. Note that the final serum dilutions, after addition of virus, run from a starting dilution of 1/4 ~~to 1/256~~. A separate control plate should include titration of both a negative and positive (high and low) horse ~~serum~~ sera of known titre, cell control (no virus), and a back titration of virus ~~control (no serum), and a virus titration~~ using six wells per log dilution (100 $TCID_{50}$ to 0.01 $TCID_{50}$/well) ~~calculate the actual amount of virus used in the test~~

vi) Incubate the plates for 1 hour at 37°C in 5% $CO_2$ atmosphere. ~~Add 50 µl of the prepared E-Derm or RK-13 cell suspension (5 × $10^5$ cells/ml) in MEM/10% FCS to each well.~~

vii) Transfer 50 µl from each well of the test and control plates to the tissue culture microtitre plates.

viii) Incubate the plates for 2 ~~4~~–5 days at 37°C in an atmosphere of 5% $CO_2$ in air.

ix) Examine the plates microscopically for CPE and record the results on a worksheet. Confirm the validity of the test by establishing that the working dilution of stock virus is at 100 $TCID_{50}$/well, that the (high and low) positive control sera are within one well of their pre-determined titre and that the negative control serum is negative at a 1/4 dilution. This takes approximately 72 hours. If at this stage the antigen is too weak the virus concentration may be increased by extending the incubation period up to 5 days. If the antigen is too strong the test must be repeated.

Wells are scored as positive for neutralisation of virus if ≥ 75% of the cell monolayer remains intact. The highest dilution of serum resulting in ≥ 75% neutralisation of virus (<25% CPE) in replicate wells is the end-point titre for that serum. Examine the plates microscopically for CPE and record the results on a worksheet.

x) Alternatively, the cell monolayers can be scored for CPE after fixing and staining as follows: after removal of the culture fluid, immerse the plates for 15 minutes in a solution containing 2 mg/ml crystal violet, 10% formalin, 45% methanol, and 45% water. Then, rinse the plates vigorously under a stream of running tap water. Wells containing intact cell monolayers stain blue, while monolayers destroyed by virus do not stain. ~~Verify that the cell control, positive serum control, and serum cytotoxicity control wells stain blue, that the virus control and negative serum control wells are not stained, and that the actual amount of virus added to each well is between $10^{1.5}$ and $10^{2.5}$ $TCID_{50}$. Wells are scored as positive for neutralisation of virus if 100% of the cell monolayer remains intact. The highest dilution of serum resulting in complete neutralisation of virus (no CPE) in both duplicate wells is the end-point titre for that serum.~~

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

## 2.2. Complement fixation test

The CFT can be used for the detection and quantification of antibodies ~~against~~ to EHV-1. The test determines whether an antigen and an antibody are capable of forming a complex. The presence of an immune complex is revealed by the detector system, which consists of guinea-pig complement and sensitised sheep red blood cells (SRBCs) coated with rabbit haemolytic serum (haemolysin). In the absence of antibodies against equine herpesvirus, no antibody/antigen complex is formed, the complement remains free in the solution and the sensitised SRBCs become lysed. In the presence of antibodies against equine herpesvirus, an antibody/antigen complex is formed, the complement becomes fixed and is therefore unable to lyse the SRBCs. They subsequently form a button at the bottom of the test well.

Guinea-pig complement, rabbit haemolytic serum, complement fixation diluent (CFD) and bovine serum albumin (BSA) can be obtained commercially. The dilution of guinea-pig complement that has activity at 3 HD (haemolytic dose) in the presence of sensitised SRBCs should be optimised. The recommended dilution of rabbit haemolytic serum (or the working dilution) is sometimes provided by the supplier. However, the optimal dilution of haemolysin should be determined with the in use reagents (complement etc.) so that the test can be performed reproducibly. The optimum concentration of antigen to be used in the test should be determined using an antigen versus antibody chequerboard technique and by testing a panel of known positive sera.

The test is performed in U bottomed microtitre plates. Paired sera should be assayed on the same plate. An antibody positive serum should be included as a control on each plate. All sera are tested on a second plate containing all components except virus to check for anti-complementary activity. A back titration of the working dilution (3 HD) of complement to 2 HD, 1 HD, 0.5 HD is set up in duplicate wells on the complement control plate (eight wells in total). An SRBC control is set up in eight wells.

### 2.2.3. Preparation of samples

i) Samples and controls are prepared by adding 4 volumes (600 µl) of CFD to 1 volume (150 µl) of test sera to give a 1/5 dilution.

ii) Diluted serum is inactivated for 30 minutes at 60°C to destroy the naturally occurring complement.

### 2.2.4. Test procedure

i) Prepare the test plate and anti-complementary plate by adding 25 µl 0.05% BSA/CFD to all wells except the first column (H).

ii) Add 50 µl of 0.05% BSA/CFD to the eight wells of the complement control (back titration).

iii) Add 75 µl of 0.05% BSA/CFD to eight wells of cell control.

iv) Add 50 µl of the diluted inactivated test serum and controls to the first well of each row on both the test and anti-complementary plates. Serial doubling dilutions are then made by transferring 25 µl across the plate and discarding the final 25 ml.

v) Place the microtitre plates on ice for addition of antigen and complement.

vi) Add 25 µl of antigen (diluted to working strength in 0.05% BSA/CFD) to the test plates.

vii) Add 25 µl of 0.05% BSA/CFD to all wells of the anti-complementary plate to compensate for lack of antigen.

viii) Add 25 µl of guinea-pig complement diluted in 0.05% BSA/CFD to 3 HD to all wells except the complement control and SRBC control.

ix) Back titrate the working dilution of 3 HD complement to 2 HD, 1 HD and 0.5 HD in 200 µl volumes. Add 25 µl of each dilution to the appropriate wells.

x) Incubate all plates at 4°C overnight.

### 2.2.5. Preparation and addition of sheep blood

i) SRBCs collected into Alsever's solution are washed twice in 0.05% BSA/PBS solution.

ii) Gently resuspend the SRBCs in 5–10 ml 0.05% BSA/CFD solution. Dilute to 2% SRBCS (v/v packed cells) in BSA/CFD solution.

~~iii) Mix the 2% SRBCs with an equal volume of BSA/CFD solution containing haemolysin at its optimal sensitising concentration to give a 1% SRBC solution. Prepare an appropriate volume of this solution by allowing 3 ml per microtitre plate.~~

~~iv) Incubate at 37°C for 10 minutes. Store the 1% sensitised SRBCs overnight at 4°C.~~

~~v) The following day, incubate the 1% sensitised SRBCs at 37°C for 30 minutes. During the final 20 minutes of this incubation, transfer the test plates from 4°C to 37°C.~~

~~vi) At the end of the 30-minute incubation, add 25 ml of 1% sensitised SRBCs to all plates. Mix on a plate shaker for 30 seconds.~~

~~vii) Incubate the plates at 37°C for 30 minutes. Shake the plates after 15 minutes and at the end of this incubation (a total of three times).~~

~~viii) Incubate the plates at 4°C for 2 hours to allow the cells to settle.~~

~~ix) Read and record the test results after 2 hours.~~

#### ~~2.2.6. Reading results~~

~~i) Confirm the validity of the test by establishing that the working dilution of complement is at 3 HD: 100% lysis at 3 HD and 2 HD, and 50% lysis at 1 HD. Distinct buttons should be visible in the eight wells of the SRBC control.~~

~~ii) There must be 100% lysis observed at the 1/5 dilution for the negative control (<5). The antibody titre of the positive control serum must read within one well of its predetermined titre.~~

~~iii) Confirm that there are no buttons visible on the anti-complementary plates. Buttoning indicates either the presence of residual native complement in the sample or that there is a non-specific complement fixing effect occurring. Sera that show anti-complementary activity should be retested and treated as described below.~~

~~iv) In the test wells, buttoning indicates the presence of antibodies in the serum. The antibody titre is the dilution at which there is 50% buttoning and 50% lysis observed.~~

#### ~~2.2.7. Treatment of samples showing anti-complementary activity~~

~~i) Add 50 µl of guinea-pig complement to 150 µl of the serum showing anti-complementary activity.~~

~~ii) Incubate the sample at 37°C for 30 minutes.~~

~~iii) Add 550 µl of CFD (1:5 dilution).~~

~~iv) Heat inactivate at 60°C for 30 minutes.~~

# C. REQUIREMENTS FOR VACCINES

## 1. Background

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.

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.

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.

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.

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

### 2.1. Characteristics of the seed

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.

#### 2.1.1. Biological characteristics of the master seed

Permanently stored stocks of both MSV and master cell stock (MCS) used for vaccine production must be demonstrated to be pure, safe and, in the case of MSV, also immunogenic.

Generally, the fifth passage from the MSV and the twentieth passage from the MCS are the highest allowed for vaccine production. Results of all quality control tests on master seeds must be recorded and made a part of the licensee's permanent records.

#### 2.1.2. Quality criteria

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

#### 2.1.3. Validation as a vaccine strain

Tests for immunogenicity of the EHV-1/4 MSV stocks should be performed in horses on an experimental test vaccine prepared from the highest passage level of the MSV allowed for use in vaccine production. The test for MSV immunogenicity consists of vaccination of horses with low antibody titres (< 1:24 by VN test) to EHV-1/4, with doses of the test vaccine that will be recommended on the final product label (Goodman *et al.*, 2006; Van de Walle *et al.*, 2010). Second serum samples should be obtained and tested for significant increases in neutralising antibody titre against the virus, 21 days after the final dose.

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

### 2.2. Method of manufacture

#### 2.2.1. Procedure

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

#### 2.2.2. Requirements for ingredients

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

#### 2.2.3. Final product batch tests

i) Sterility

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

ii) Identity

Identity tests shall demonstrate that no other vaccine strain is present when several strains are propagated in a laboratory used in the production of multivalent vaccines.

iii) Safety

Safety tests shall consist of detecting any abnormal local or systemic adverse reactions to the vaccine in the host species by all vaccination route(s). Tests to assure safety of each production batch of ER vaccine must demonstrate complete inactivation of virus (for inactivated vaccines) as well as a level of residual virus-killing agent that does not exceed the maximal allowable limit (e.g. 0.2% for formaldehyde).

iv) Batch potency

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

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

#### 2.3.1. Manufacturing process

For registration of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Sections C.2.1 and C.2.2) should be submitted to the authorities. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

#### 2.3.2 Safety requirements

Vaccine safety should be evaluated in vaccinated animals using different assays (see Section 2.2.3.iii).

#### 2.3.3 Efficacy requirements

Vaccine efficacy (protection) is estimated in vaccinated animals directly by evaluating their resistance to live pathogen challenge.

#### 2.3.4 Duration of immunity

As part of the licensing or marketing authorisation procedure, the manufacturer may be required to demonstrate the duration of immunity (DOI) of a given vaccine by either challenge or alternative test at the end of the claimed period of protection.

631 Tests to establish the duration of immunity to EHV-1/4 or EHV1/4 achieved by immunisation with
632 each batch of vaccine are not required. The results of many reported observations indicate that
633 immunity induced by vaccination-against EHV-1 or EHV induced immunity to EHV-1/4 is not more
634 than a few months in duration; these observations are reflected in the frequency of revaccination
635 recommended on ER vaccine product labels.

636 ### 2.3.5 Stability

637 As part of the licensing or marketing authorisation procedure, the manufacturer will be required
638 to demonstrate the stability of all the vaccine's properties at the end of the claimed shelf-life
639 period. Storage temperature shall be indicated, and warnings should be given if product is
640 damaged by freezing or ambient temperature.

641 At least three production batches of vaccine should be tested for shelf life before reaching a
642 conclusion on the vaccine's stability. When stored at 4°C, inactivated vaccine products generally
643 maintain their original antigenic potency for at least 1 year. Lyophilised preparations of the live
644 virus vaccine are also stable during storage for 1 year at 4°C. Following reconstitution, live virus
645 vaccine is unstable and cannot be stored without loss of potency.

646 **Note:** current vaccines are authorised for prevention of respiratory disease or as an aid in the prevention of abortion.
647 Unless the vaccine's ability to prevent neurological disease is under investigation, the virus used in the challenge
648 experiments should not be a strain with a history of inducing neurological disease.

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807 *
808 * *

809 **NB:** There are WOAH Reference Laboratories for equine rhinopneumonitis (please consult the WOAH Web site:
810 https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3http://www.oie.int/).
811 Please contact the WOAH Reference Laboratories for any further information on
812 diagnostic tests, reagents and vaccines for equine rhinopneumonitis
813 and to submit strains for further characterisation.

814 **NB:** FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2017.

**MEETING OF THE BIOLOGICAL STANDARDS COMMISSION**

**Paris, 5–9 February 2024**

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# SECTION 3.8.

# ~~OVIDAE AND~~ CAPRINAE

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# 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 tissues of aborted or stillborn lambs is often difficult but virus can be detected by sensitive reverse transcriptase polymerase chain reaction methods that are able to detect residual nucleic acid. However, tissues and blood from PI sheep more than a few months old contain high levels of virus, which can be easily identified by isolation and direct methods to detect antigens or nucleic acids. As sheep may be infected with BVDV, it is preferable to use diagnostic assays that are 'pan-pestivirus' reactive and will readily detect all strains of BDV and BVDV.*

***Serological tests:*** *Acute infection with BDV is best confirmed by demonstrating seroconversion using paired or sequential samples from several animals in the group. The enzyme-linked immunosorbent assay and virus neutralisation test (VNT) are the most commonly used antibody detection methods. Due to the antigenic differences between BDV and BVDV, assays for the detection of antibodies to BDV, especially by VNT, should preferably be based on a strain of BDV.*

***Requirements for vaccines:*** *There is no standard vaccine for BDV, but a commercial killed whole-virus vaccine has been produced. Ideally, such a vaccine should be suitable for administration to females before breeding for prevention of transplacental infection. The use of BVDV vaccines has been advocated, but the antigenic diversity of BD viruses must be considered. In many instances, the antigenic diversity of BDV strains is sufficiently different to BVDV that a BVDV vaccine is unlikely to provide protection.*

*BD viruses have contaminated several modified live veterinary vaccines produced in sheep cells or containing sheep serum. This potential hazard should be recognised by manufacturers of biological products.*

## A. INTRODUCTION

Border disease virus (BDV) is a *Pestivirus* of the family *Flaviviridae* and is closely related to classical swine fever virus (CSFV) and bovine viral diarrhoea virus (BVDV). There are ~~four~~ a number of officially recognised species, namely – BDV (*Pestivirus ovis*) CSFV (*Pestivirus suis*), BVDV types 1 and 2 (taxonomically known as *Pestivirus bovis* and *Pestivirus tauri*, respectively) and ~~BDV (ICTV, 2016)~~ BVDV 3 or Hobi-like pestivirus (*Pestivirus brazilense*) (Postler *et al.*, 2023), but a number of other pestiviruses that are considered to be distinct species have been reported. While CSF viruses are predominantly restricted to pigs, ~~examples of~~ there are situations where the other ~~three~~ species have ~~all~~ been recovered from sheep. While the majority of isolates have been identified as BD viruses in areas where sheep or goats are raised in isolation from other species (Vilcek *et al*., 1997), in regions where there is close contact between small ruminants and cattle, BVDV may be frequently identified (Carlsson, 1991). Nearly all virus isolates of BDV are noncytopathogenic, although occasional cytopathic viruses have been isolated (Vantsis *et al*., 1976). BDV spreads naturally among sheep by the oro-nasal route and by vertical transmission. It is principally a cause of congenital disease in sheep and goats, but can also cause acute and persistent infections. Infection is less common in goats, in which persistent infection is rare as abortion is the main presenting sign. Pigs may also be infected by pestiviruses other than CSFV and antibodies to BDV in pigs may interfere with tests for the diagnosis of CSF (Oguzoglu *et al*., 2001). Several genotypes of BD viruses from sheep, goats and Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*) have been described. Phylogenetic analysis using computer-assisted nucleotide sequence analysis suggests that genetic variability among BD viruses is greater than within each of the other *Pestivirus* species. ~~Four distinguishable genogroups of BDV have been described as well as putative novel *Pestivirus* genotypes from Tunisian sheep and a goat~~ At least eight BDV genotypes have been described (BDV type 1 to BDV type 8). Other ovine pestiviruses have been identified that are responsible for BD-like syndromes such as Tunisian and Tunisian-like, Aydin-like (Pestivirus I, Turkey) Pestivirus genotypes from Tunisian sheep and a goat and a new emerging ovine pestivirus (OVPV) that were found to be genetically and antigenically closely related to CSFV (Becher *et al*., 2003; Righi *et al*., 2021; Vilcek & Nettleton, 2006). The chamois BDV is similar to isolates from sheep in the Iberian Peninsula (Valdazo-Gonzalez *et al*., 2007). This chapter describes BDV infection in sheep. Chapter 3.4.7 *Bovine viral diarrhoea* should also be consulted for related diagnostic methods.

### 1. Acute infections

Healthy newborn and adult sheep exposed to BDV usually experience only mild or inapparent disease. Slight fever and a mild leukopenia are associated with a short-lived viraemia detectable between days 4 and 11 post-infection, after which virus neutralising antibody appears in the serum (Thabti *et al*., 2002).

Acute infections are best diagnosed serologically using paired sera from a representative number of sheep. Occasional BDV isolates have been shown to produce high fever, profound and prolonged leukopenia, anorexia, conjunctivitis, nasal discharge, dyspnoea and diarrhoea, and 50% mortality in young lambs. One such isolate was recovered from a severe

87 epidemic of BD among dairy sheep in 1984 (Chappuis *et al*., 1986). A second such isolate was a BDV contaminant of a
88 live CSFV vaccine (Wensvoort & Terpstra, 1988).

## 89 2. Fetal infection

90 The main clinical signs of BD are seen following the infection of pregnant ewes. While the initial maternal infection is
91 subclinical or mild, the consequences for the fetus are serious. Fetal death may occur at any stage of pregnancy, but is
92 more common in fetuses infected early in gestation. Small dead fetuses may be resorbed or their abortion may pass
93 unnoticed as the ewes continue to feed well and show no sign of discomfort. As lambing time approaches, the abortion of
94 larger fetuses, stillbirths and the premature births of small, weak lambs will be seen. Confirmation that an abortion or
95 stillbirth is due to BDV is often difficult to establish, but virus may be isolated from fetal tissues in some cases. The use of
96 an appropriate real-time reverse-transcription polymerase chain reaction (RT-PCR) assay may give a higher level of
97 success because of the advantages of high sensitivity and the ability to detect genome from non-infectious virus. In aborted
98 fetuses, it is also possible to detect virus by immunohistochemistry of brain, thyroid and other tissues (Thur *et al*., 1997).
99 Samples of fetal fluids or serum should be tested for BDV antibody.

100 During lambing, an excessive number of barren ewes will become apparent, but it is the diseased live lambs that present
101 the main clinical features characteristic of BD. The clinical signs exhibited by BD lambs are very variable and depend on
102 the breed of sheep, the virulence of the virus and the time at which infection was introduced into the flock. Affected lambs
103 are usually small and weak, many being unable to stand. Nervous signs and fleece changes are often apparent. The
104 nervous signs of BD are its most characteristic feature. The tremor can vary from violent rhythmic contractions of the
105 muscles of the hindlegs and back, to barely detectable fine trembling of the head, ears, and tail. Fleece abnormalities are
106 most obvious in smooth-coated breeds, which develop hairy fleeces, especially on the neck and back. Abnormal brown or
107 black pigmentation of the fleece may also be seen in BD-affected lambs. Blood samples to be tested for the presence of
108 BDV or antibody should be collected into anticoagulant from suspect lambs before they have received colostrum. Once
109 lambs have ingested colostrum, it is difficult to isolate virus until they are 2 months old and maternal antibody levels have
110 waned. However, during this period, it may be possible to detect viral antigen in skin biopsies, by immunohistochemistry,
111 in washed leukocytes by enzyme-linked immunosorbent assay (ELISA) or by real-time RT-PCR. ELISAs directed at
112 detection of the Erns antigen appear to be less prone to interference by maternal antibodies and can often be used to
113 detect antigen in serum.

114 With careful nursing, a proportion of BD lambs can be reared, although deaths may occur at any age. The nervous signs
115 gradually decline and can disappear by 3–6 months of age. Weakness, and swaying of the hind-quarters, together with
116 fine trembling of the head, may reappear at times of stress. Affected lambs often grow slowly and under normal field
117 conditions many will die before or around weaning time. In cases where losses at lambing time have been low and no
118 lambs with obvious signs of BD have been born, this can be the first presenting sign of disease.

119 Some fetal infections occurring around mid-gestation can result in lambs with severe nervous signs, locomotor
120 disturbances and abnormal skeletons. Such lambs have lesions of cerebellar hypoplasia and dysplasia, hydranencephaly
121 and porencephaly resulting from necrotising inflammation. The severe destructive lesions appear to be immune mediated,
122 and lambs with such lesions frequently have high titres of serum antibody to BDV. Most lambs infected in late gestation
123 are normal and healthy and are born free from virus but with BDV antibody. Some such lambs can be weak and may die
124 in early life (Barlow & Patterson, 1982).

## 125 3. Persistent viraemia

126 When fetuses survive an infection that occurs before the onset of immune competence, they are born with a persistent
127 viraemia. The ovine fetus can first respond to an antigenic stimulus between approximately 60 and 85 days of its 150-day
128 gestation period. In fetuses infected before the onset of immune competence, viral replication is uncontrolled and 50%
129 fetal death is common. In lambs surviving infection in early gestation, virus is widespread in all organs. Such lambs appear
130 to be tolerant of the virus and have a persistent infection, usually for life. A precolostral blood sample will be virus positive
131 and antibody negative. Typically, there is no inflammatory reaction and the most characteristic pathological changes are
132 in the central nervous system (CNS) and skin. Throughout the CNS, there is a deficiency of myelin, and this causes the
133 nervous signs. In the skin, primary wool follicles increase in size and the number of secondary wool follicles decreases,
134 causing the hairy or coarse fleece.

135 Persistently viraemic sheep can be identified by the detection of viral antigens, nucleic acids or infectious virus in a blood
136 sample. Viraemia is readily detectable by testing of serum at any time except within the first 2 months of life, when virus
137 may be masked by colostral antibody and, possibly, in animals older than 4 years, some of which develop low levels of
138 anti-BDV antibody (Nettleton *et al*., 1992). Methods other than virus isolation may be preferred to avoid interference from
139 antibodies. When the presence of colostral antibodies is suspected, the virus may be detected in washed leukocytes and
140 in skin by using sensitive ELISAs. Although virus detection in blood during an acute infection is difficult, persistent viraemia
141 should be confirmed by retesting animals after an interval of at least 3 weeks. The use of real-time RT-PCR should be

considered at all times and for any sample type due to its high analytical sensitivity and the lack of interference from antibodies in a sample.

Some viraemic sheep survive to sexual maturity and are used for breeding. Lambs born to these infected dams are always persistently viraemic. Persistently viraemic sheep are a continual source of infectious virus to other animals and their identification is a major factor in any control programme. Sheep being traded should be screened for the absence of BDV viraemia.

Usually persistently infected (PI) rams have poor quality, highly infective semen and reduced fertility. All rams used for breeding should be screened for persistent BDV infection on a blood sample. Semen samples can also be screened for virus, but virus isolation is much less satisfactory than from blood because of the toxicity of semen for cell cultures. Real-time RT-PCR for detection of pestivirus nucleic acid would usually overcome toxicity problems, and thus this assay should be useful for testing semen from rams.

## 4. Late-onset disease in persistently viraemic sheep

Some PI sheep housed apart from other animals spontaneously develop intractable diarrhoea, wasting, excessive ocular and nasal discharges, sometimes with respiratory distress. At necropsy such sheep have gross thickening of the distal ileum, caecum and colon resulting from focal hyperplastic enteropathy. Cytopathic BDV can be recovered from the gut of these lambs. With no obvious outside source of cytopathic virus, it is most likely that such virus originates from the lamb's own virus pool, similar to what occurs with BVDV. Other PI sheep in the group ~~do~~ may not develop the disease. This syndrome, which has been produced experimentally and recognised in occasional field outbreaks of BD, has several similarities with bovine mucosal disease (Nettleton *et al*., 1992).

# B. DIAGNOSTIC TECHNIQUES

***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 | +++ | +++ | +++ | +++ | +++ | – |
| 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.

168 ## 1. Identification of the agent

169 There is no designated WOAH Reference Laboratory for BDV, but the reference laboratories for BVDV or CSFV will be
170 able to provide advice[1]. One of the most sensitive proven methods for identifying BDV remains virus isolation. However, a
171 broadly reactive real-time RT-PCR assay (preferably pan-pestivirus reactive) will usually provide higher analytical
172 sensitivity than virus isolation, can be used to test samples that are difficult to manage by virus isolation and can be
173 performed in a few hours. Antigen-detection ELISA and immunohistochemical techniques on tissue sections are also
174 valuable methods for identifying BDV-infected animals.

175 ### 1.1. Virus isolation

176 It is essential that laboratories undertaking virus isolation have a guaranteed supply of pestivirus-free
177 susceptible cells and bovine serum, or equivalent, that contain no anti-pestivirus activity and no contaminating
178 virus. It is important that a laboratory quality assurance programme be in place. Chapter 3.4.7 provides detailed
179 methods for virus isolation in either culture tubes or microplates for the isolation of pestiviruses from sheep or
180 goat samples, including serum, whole blood, semen and tissues. The principles and precautions outlined in that
181 chapter for the selection of cell cultures, medium components and reagents are equally relevant to this chapter.
182 Provided proven pan-pestivirus reactive reagents (e.g. monoclonal antibodies [MAbs], primers and probes for
183 real-time RT-PCR) are used for antigen or nucleic acid detection, the principal difference is the selection of
184 appropriate cell cultures.

185 BD virus can be isolated in a number of primary or secondary ovine cell cultures (e.g. kidney, testes, lung).
186 Ovine cell lines for BDV growth are rare. Semicontinuous cell lines derived from fetal lamb muscle (FLM), whole
187 embryo (Thabti *et al*., 2002) or sheep choroid plexus can be useful, but different lines vary considerably in their
188 susceptibility to the virus. Ovine cells have been used successfully for the isolation and growth of BD viruses
189 and BVDV types 1 and 2 from sheep. In regions where sheep may become infected with BVD viruses from
190 cattle, a virus isolation system using both ovine and bovine cells could be optimal. However, bovine cells have
191 lower sensitivity for the primary isolation and growth of some BD viruses, so reliance on bovine cells alone is
192 inadvisable. Details of suitable bovine cell cultures are provided chapter 3.4.7. The precautions outlined in that
193 chapter for the establishment of cells and medium components that are free from contamination with either
194 pestiviruses or antibodies, and measures to ensure that the cells are susceptible to a wide range of local field
195 strains are equally relevant to systems for detection of BDV.

196 From live animals, serum is the most frequently used sample to be tested for the presence of infectious virus.
197 However, for difficult cases, the most sensitive way to confirm pestivirus viraemia is to wash leukocytes
198 repeatedly (at least three times) in culture medium before co-cultivating them with susceptible cells in either cell
199 culture tubes or microplates. After culture for 5–7 days, the cultures should be frozen and thawed once and an
200 aliquot of diluted culture fluid passaged onto further susceptible cells grown in microplates or on chamber slides
201 to allow antigen detection by immunocytochemistry. Staining for noncytopathic pestiviruses will usually detect
202 virus at the end of the primary passage, but to detect slow-growing viruses in poorly permissive cells two
203 passages are desirable. It is recommended that the culture supernatant used as inoculum for the second
204 passage is diluted approximately 1/100 in new culture medium because some high titred field isolates will
205 replicate poorly if passaged undiluted (i.e. at high multiplicity of infection – moi).

206 Tissues should be collected from dead animals in virus transport medium. In the laboratory, the tissues are
207 ground to give a 10–20% (w/v) suspension, centrifuged to remove debris, and the supernatant passed through
208 0.45 µm filters. Spleen, lung, thyroid, thymus, kidney, brain, lymph nodes and gut lesions are the best organs
209 for virus isolation.

210 Semen can be examined for the presence of BDV, but raw semen is strongly cytotoxic and must be diluted,
211 usually at least 1/10 in culture medium. As the major threat of BDV-infected semen is from PI rams, blood is a
212 more reliable clinical sample than semen for identifying such animals. There are many variations in virus
213 isolation procedures. All should be optimised for maximum sensitivity using a standard reference virus
214 preparation and, whenever possible, recent BDV field isolates. Most of the limitations of virus isolation for the
215 detection of BDV in serum or blood, tissues or semen can be overcome by the use of a proven, sensitive pan-
216 pestivirus reactive real-time RT-PCR. Some laboratories screen samples by real-time RT-PCR and undertake
217 virus isolation on positive samples to collect BDV strains for future reference or research purposes.

218 For specific technical details of virus isolation procedures, including immunoperoxidase staining, refer to chapter
219 3.4.7.

[1] Please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

### 1.2. Nucleic acid detection methods

The complete genomic sequences of three BD viruses have been determined and compared with those of other pestiviruses (Becher *et al*., 1998; Ridpath & Bolin, 1997). Phylogenetic analysis shows BD viruses to be more closely related to CSFV than to BVDV (Becher *et al*., 2003; Van Rijn *et al*., 1997; Vilcek & Nettleton, 2006; Vilcek *et al*., 1997). Real-time RT-PCR for diagnosing pestivirus infection is now used widely and a number of formats have been described. Real-time RT-PCR assays have the advantages of being able to detect both infectious virus and residual nucleic acid, the latter being of value for investigating abortions and lamb deaths. Furthermore, the presence of virus-specific antibodies in a sample will have no adverse effect on the sensitivity of the real-time RT-PCR assay. These assays are also useful for screening semen and, when recommended nucleic acid extraction protocols are followed, are less affected by components of the semen compared with virus isolation. Because of the potential for small ruminants to be infected with genetically different strains of BDV or with strains of BVDV, a ~~proven~~ pan-pestivirus reactive real-time RT-PCR with proven high sensitivity should be used. To ensure that the genetic spectrum of BDV strains is sufficiently covered, it may be necessary to apply a broadly reactive BDV specific real time RT-PCR in parallel to maximise diagnostic sensitivity. Suitable protocols for both nucleic acid extraction as well as the real-time RT-PCR are described in chapter 3.4.7. All precautions to minimise laboratory contamination should be followed closely.

After testing samples in a pan-pestivirus reactive assay, samples giving ~~positive results can~~ any level of reactivity should be investigated further by the application of a BDV-specific real-time RT-PCR (Willoughby *et al*., 2006). It is important to note however that different genotypes of BDV may be circulating in some populations, especially wild ruminants such as chamois and deer, and may be transferred to sheep. An assay that is specific for the detection of BDV should be used with some caution as variants or previously unrecognised genotypes may not be detected, hence the value of initially screening samples with a pan-pestivirus reactive real-time RT-PCR. Nevertheless, there are also situations where a pan-pestivirus reactive real-time RT-PCR may have lower analytical sensitivity. Consequently, in any situation where BDV infection is suspected, the application of several diagnostic methods is recommended. Maternal serology can also play an important role as negative results should exclude the potential involvement of a pestivirus.

### 1.3. Enzyme-linked immunosorbent assay for antigen detection

ELISAs for the direct detection of pestivirus antigen in blood and tissues of infected animals have proven to be extremely useful for the detection of PI animals and the diagnosis of disease. The first ELISA for pestivirus antigen detection was described for detecting viraemic sheep and was later modified into a double MAb capture ELISA for use in sheep and cattle (Entrican *et al.,* 1994). The test is most commonly employed to identify PI viraemic sheep using washed, detergent-lysed blood leukocytes. The sensitivity is close to that of virus isolation and it is a practical method for screening large numbers of blood samples. As with virus isolation, high levels of colostral antibody can mask persistent viraemia. The ELISA is more effective than virus isolation in the presence of antibody, but may give false-negative results in viraemic lambs younger than 2 months old. The ELISA is usually not sensitive enough to detect acute BDV infections on blood samples. As well as for testing leukocytes, the antigen ELISA can also be used on tissue suspensions, especially spleen, from suspected PI sheep and, as an alternative to immunofluorescence and immunoperoxidase methods, on cell cultures. Several pestivirus ELISA methods have been published but there are at present no commercially available kits that have been fully validated for detecting BDV. Prior to use for regulatory purposes, these kits should be validated in the region where they are to be used to ensure that a wide range of field strains of BDV can be detected and that they are suitable for the sample types to be tested.

### 1.4. Immunohistochemistry

Viral antigen demonstration is possible in most of the tissues of PI animals (Braun *et al*., 2002; Thur *et al*., 1997) although this is not a method that is routinely used for diagnostic purposes. This should be done on acetone-fixed frozen tissue sections (cryostat sections) or paraffin wax embedded samples using appropriate antibodies. Pan-pestivirus reactive antibodies with NS2-3 specificity are suitable. Tissues with a high amount of viral antigen are brain, thyroid gland, lung and oral mucosa. Skin biopsies have been shown to be useful for *in-vivo* diagnosis of persistent BDV infection.

## 2. Serological tests

Antibody to BDV is usually detected in sheep sera using VN or an ELISA. The less sensitive agar gel immunodiffusion test is not recommended. Control positive and negative reference sera must be included in every test. These should give results within predetermined limits for the test to be considered valid. Single sera can be tested to determine the prevalence of BDV in a flock, region or country. For diagnosis, however, acute and convalescent sera are the best samples for confirming

acute BDV infection. Repeat sera from one animal should always be tested alongside each other on the same plate to provide a reliable comparison of titres.

## 2.1. Virus neutralisation test

Due to antigenic diversity among pestiviruses the choice of test virus is difficult (Dekker *et al*., 1995; Nettleton *et al*., 1998). No single strain of BDV is ideal. A local strain that gives the highest antibody titre with a range of positive sheep sera should be used.

Because there are few cytopathogenic strains of BDV available, to achieve optimal analytical sensitivity, it is more usual to employ a representative local non-cytopathogenic strain and read the assay after immunoperoxidase staining of the cells. Proven highly sensitive, pestivirus-free sheep cells such as lamb testis or kidney cells are suitable and can be maintained as cryogenically frozen stocks for use over long periods of time. The precautions outlined for selection of pestivirus-free medium components are equally applicable to reagents to be used in VN tests. A recommended procedure follows.

### 2.1.1. Test procedure

i) The test sera are heat-inactivated for 30 minutes at 56°C.

ii) From a starting dilution of 1/4, serial twofold dilutions of the test sera are made in a cell-culture grade flat-bottomed 96-well microtitre plate, using cell culture medium as diluent. For each sample, three or four wells are used at each dilution depending on the degree of precision required. Also, for each sample and at each serum dilution, one well is left without virus to monitor for evidence of sample toxicity that could mimic viral cytopathology or interfere with virus replication. Control positive and negative sera should also be included in each batch of tests.

iii) An equal volume (e.g. 50 µl) of a stock of BDV containing 100 $TCID_{50}$ (50% tissue culture infective dose) is added to each well. A back titration of virus stock is also done in some spare wells to check the potency of the virus (acceptance limits 30 ~~80~~–300 $TCID_{50}$).

iv) The plate is incubated for 1 hour at 37°C.

v) A flask of suitable cells (e.g. ovine testis or kidney cells) is trypsinised and the cell concentration is adjusted to 2 × $10^5$/ml. 100 µl of the cell suspension is added to each well of the microtitre plate.

vi) The plate is incubated at 37°C for 4–5 days, either in a 5% $CO_2$ atmosphere or with the plate sealed.

vii) The wells are examined microscopically to ensure that there is no evidence of toxicity or cytopathic effect (CPE), then fixed and stained by immunoperoxidase staining using an appropriate MAb. The VN titre for each serum is the dilution at which the virus is neutralised in 50% of the wells. This can be calculated by the Spearman–Kärber or Reed Muench methods. A seronegative animal will show no neutralisation at the lowest dilution of serum (i.e. 1/4), equivalent to a final dilution of 1/8. For accurate comparison of antibody titres, and particularly to demonstrate significant (more than fourfold) changes in titre, samples should be tested in parallel in the same test.

viii) Occasionally there may be a need to determine whether antibody in a flock is against a virus belonging to a particular *Pestivirus* serogroup. A differential VN test can be used in which sera are titrated out against representative viruses from each of the four *Pestivirus* groups, i.e. BDV, BVDV types 1 and 2, and CSFV. Maximum titre will identify the infecting serotype and the spectrum of cross-reactivity with the other serotypes will also be revealed.

## 2.2. Enzyme-linked immunosorbent assay

An MAb-capture ELISA for measuring BDV antibodies has been described. Two pan-pestivirus MAbs that detect different epitopes on the immunodominant nonstructural protein NS 2/3 are used to capture detergent-lysed cell-culture grown antigen. The results correlate qualitatively with the VN test (Fenton *et al*., 1991).

### 2.2.1. Antigen preparation

Use eight 225 $cm^2$ flasks of newly confluent FLM cells; four flasks will be controls and four will be infected. Wash the flasks and infect four with a 0.01–0.1 m.o.i. of Moredun cytopathic BDV. Allow the virus to adsorb for 2 hours at 37°C. Add maintenance media containing 2% FBS (free from BDV antibody), and incubate cultures for 4–5 days until CPE is obvious. Pool four control flask supernatants and separately pool four infected flask supernatants. Centrifuge at 3000 ***g*** for 15 minutes to pellet cells. Discard the supernatants. Retain the cell pellets. Wash the flasks with 50 ml of PBS and repeat the centrifugation step as above. Pool all the control cell pellets in 8 ml PBS containing 1% Nonidet P40 and return 2 ml to each control flask to lyse the remaining attached cells. Repeat for infected cells. Keep the flasks at 4°C

for at least 2 hours agitating the small volume of fluid on the cells vigorously every 30 minutes to ensure total cell detachment. Centrifuge the control and infected antigen at 12,000 ***g*** for 5 minutes to remove the cell debris. Supernatant antigens are stored at –70°C in small aliquots.

#### 2.2.2. Test procedure

i) The two MAbs are diluted to a predetermined dilution in 0.05 M bicarbonate buffer, pH 9.6. All wells of a suitable ELISA-grade microtitre plate (e.g. Nunc maxisorb, Greiner 129b) are coated overnight at 4°C.

ii) After washing three times in PBST, a blocking solution of PBST containing 10% horse serum (PBSTH) is added to all wells, which are incubated at 37°C for 1 hour.

iii) The antigen is diluted to a predetermined dilution in PBSTH and alternate rows of wells are coated with virus and control antigens for 1 hour at 37°C. The plates are then washed three times in PBST before addition of test sera.

iv) Test sera are diluted 1/50 in PBSTH and added to duplicate virus and duplicate control wells for 1 hour at 37°C. The plates are then washed three times in PBST.

v) Anti-ovine IgG peroxidase conjugate is diluted to a predetermined dilution in PBSTH and added to all wells for 1 hour at 37°C. The plates are washed three times in PBST.

vi) A suitable activated enzyme substrate/chromogen, such as ortho-phenylene diamine (OPD) or tetramethyl benzidine (TMB), is added). After colour development, the reaction is stopped with sulphuric acid and the absorbance read on an ELISA plate reader. The mean value of the two control wells is subtracted from the mean value of the two virus wells to give the corrected absorbance for each serum. Results are expressed as corrected absorbance with reference to the corrected absorbance of known positive and negative sera. Alternatively, ELISA titres can be extrapolated from a standard curve of a dilution series of a known positive reference serum.

If antigens of sufficient potency can be produced the MAb capture stage can be omitted. In this case alternate rows of wells are coated with virus and control antigen diluted to a predetermined dilution in 0.05 M bicarbonate buffer, pH 9.6, overnight at +4°C. The plates are washed and blocked as in step ii above. After washing, diluted test sera are added and the test proceeds from step iv as above.

# C. REQUIREMENTS FOR VACCINES

## 1. Background

To be useful, a BDV vaccine should be effective when administered to female sheep before breeding to prevent transplacental infection. Experimental and commercial inactivated whole virus BDV vaccines have been produced in Europe (Brun *et al*., 1993; Vantsis *et al*., 1980). Unlike vaccines for BVDV, there is limited demand for vaccines against BDV and those produced have only been inactivated products. No live attenuated or recombinant subunit vaccines for BDV have been produced commercially.

Pestivirus contaminants of modified live virus vaccines have been found to be a cause of serious disease following their use in pigs, cattle, sheep and goats. Contaminated vaccines have included those used for the control of Aujesky's disease, CSF, rotavirus, coronavirus, rinderpest, sheep pox and contagious pustular dermatitis. The insidious ability of pestiviruses to cross the placenta, and thus establish PI animals, gives them the potential to contaminate vaccines through cells, serum used as medium supplement, or seed stock virus. As nearly all isolates of pestiviruses are noncytopathic, they will remain undetected unless specific tests are carried out. Although such contamination should be less likely to be a problem with an inactivated vaccine, nevertheless steps should be taken to ensure that materials used in production are not contaminated.

### 1.1. Characteristics of a target product profile

Traditionally, pestivirus vaccines fall into two classes: modified live or inactivated virus vaccines. The essential requirement for both types is to ~~afford~~ provide a high level of fetal infection. Only inactivated vaccines have been produced for BDV. Properly formulated inactivated vaccines are very safe to use but, to obtain satisfactory levels of immunity, they usually require booster vaccinations, which may be inconvenient. Because of the propensity for antigenic variability, the vaccine should contain strains of BDV that are closely matched to viruses found in the area in which they are used. This may present particular challenges with BDV in regions where several antigenic types have been found. Due to the need to customise vaccines for the most commonly encountered

strains within a country or region, it is not feasible to produce a vaccine antigen bank that can be drawn upon globally.

Guidance for the production of veterinary vaccines is given in Chapter 1.1.8 *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.

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

### 2.1. Characteristics of the seed

An ideal vaccine should contain a strain or strains of virus that give protection against all sheep pestiviruses. This may be challenging however, because of the range of pestiviruses with which sheep can be infected. There is considerable antigenic variation across these viruses – both between viruses that have been classified in the BDV genogroup as well as between viruses in the BVDV1 and BVDV2 genotypes (Becher *et al*., 2003; Vilcek & Nettleton, 2006; Wensvoort *et al*., 1989). Infection of sheep with the putative BVDV-3 genotype has also been described (Decaro *et al.,* 2012). It is likely that the antigenic composition of a vaccine will vary from region to region to provide an adequate antigenic match with dominant virus strains. Cross-neutralisation studies are required to establish optimal combinations. Nevertheless, it would appear that any BDV vaccine should contain at least a representative of the BDV and BVDV (type 1) groups. Characterisation of the biologically cloned vaccine viruses should include typing with MAbs and genotyping (Paton *et al*., 1995).

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

It is crucial to ensure that all materials used in the preparation of the bulk antigens have been extensively screened to ensure freedom from extraneous agents. This should include master and working seeds, the cell cultures and all medium supplements such as bovine serum. Some bovine viruses and particularly BVDV can readily infect small ruminants such as sheep. Therefore, it is particularly important to ensure that any serum used that is of bovine origin is free of both adventitious BVDV and antibodies against BVDV strains because low levels of either virus or antibody can mask the presence of the other. Materials and vaccine seeds should be tested for sterility and freedom from contamination with other agents, especially viruses as described in the chapter 1.1.8 and Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*.

If a vaccine passes basic tests, the efficacy of vaccination should ultimately be measured by the capacity to prevent transplacental transmission. Effective challenge of vaccinated pregnant ewes at 50–60 days gestation has been achieved by intranasal installation of virus or by mixing with PI sheep (Brun *et al.,* 1993). Usually this reliably produces persistently viraemic offspring in non-immune ewes. In regions where multiple genotypes of BDV viruses are commonly encountered, efficacy in protecting against multiple strains should be measured.

### 2.2. Method of manufacture

#### 2.2.1. Procedure

Inactivated vaccines have been prepared using conventional laboratory techniques with stationary or rolled cell cultures. Inactivants have included formalin and beta-propriolactone. Adjuvants have included aluminium hydroxide and oil (Brun *et al*., 1993; Vantsis *et al*., 1980). Optimal yields depend on the cell type and isolate used. A commercial BDV vaccine containing two strains of virus has been prepared on ovine cell lines (Brun *et al*., 1993). Cells must be produced according to a seed-lot system from a master cell seed (MCS) that has been shown to be free from all contaminating microorganisms. Vaccine should only be produced in cells fewer than 20 passages from the MCS. Control cells from every passage should be checked for pestivirus contamination. Standard procedures may be used, with the expectation for harvesting noncytopathic virus on days 4–7 after inoculation of cultures. The optimal yield of infectious virus will depend on several factors, including the cell culture, isolate used and the initial seeding rate of virus. These factors should be taken into consideration and virus replication kinetics investigated to establish the optimal conditions for large-scale virus production. Whether a live or inactivated vaccine, the essential aim will be to produce a high-titred virus stock. This bulk antigen preparation can subsequently be prepared according to the type of vaccine being considered.

#### 2.2.2. Requirements for ingredients

BDV vaccines have usually been grown in cell cultures of ovine origin that are frequently supplemented with medium components of animal origin. The material of greatest concern is bovine serum due to the

potential for contamination with BVD viruses and antibodies to these viruses. These adventitious contaminants not only affect the efficiency of production but also may mask the presence of low levels of infectious BVDV that may have undesirable characteristics. In addition to the virus seeds, all materials should be tested for sterility and freedom from contamination with other agents, especially viruses as described in chapters 1.1.8 and 1.1.9. Furthermore, materials of bovine or ovine origin should originate from a country with negligible risk for transmissible spongiform encephalopathies (see chapter 1.1.9).

### 2.2.3. In-process controls

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

### 2.2.4. Final product batch tests

i) Sterility

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

ii) Identity

Identity tests should demonstrate that no other strain of BDV is present when several strains are propagated in a facility producing multivalent vaccines.

iii) Safety

Samples from inactivated vaccines should be tested rigorously for viable virus. Samples of the product should be passaged for a minimum of three passages in sensitive cell cultures to ensure absence of live BDV. This *in-vitro* monitoring can be augmented by injecting two BDV-seronegative sheep with 20 doses of unformulated antigen as part of a standard safety test. Presence of live virus will result in the development of a more convincing serological response than will occur with inactivated virus alone. The sheep sera can also be examined for antibody to other prescribed agents.

Safety tests shall also consist of detecting any abnormal local or systemic adverse reactions to the vaccine by all vaccination route(s). Batch-to-batch safety tests are required unless safety of the product is demonstrated and approved in the registration dossier and production is consistent with that described in chapter 1.1.8. Vaccines must either be demonstrated to be safe in pregnant sheep (i.e. no transmission to the fetus), or should be licensed with a warning not to use them in pregnant animals.

iv) Batch potency

Vaccine potency is best tested in seronegative sheep in which the development and level of antibody is measured. BVD vaccines must be demonstrated to produce adequate immune responses when used in their final formulation according to the manufacturer's published instructions. The minimum quantity of infectious virus or antigen required to produce an acceptable immune response should be determined. An indirect measure of potency is given by the level of virus infectivity prior to inactivation. *In-vitro* assays should be used to monitor individual batches during production. The antigen content following inactivation can be assayed by MAb-capture ELISA and related to the results of established *in-vivo* potency results. It should be demonstrated that the lowest recommended dose of vaccine can prevent transplacental transmission of BDV in pregnant sheep.

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

#### 2.3.1. Manufacturing process

For registration of a vaccine, all relevant details concerning manufacture of the vaccine and quality control testing should be submitted to the relevant authorities. Unless otherwise specified by the authorities, information should be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

There is no standard method for the manufacture of a BDV vaccine, but conventional laboratory techniques with stationary, rolled or suspension (micro-carriers) cell cultures may be used. Inactivated vaccines can be prepared by conventional methods, such as binary ethylenimine, formalin or beta-propiolactone inactivation (Park & Bolin, 1987). A variety of adjuvants may be used.

#### 2.3.2. Safety requirements

*In-vivo* tests should be undertaken using repeat doses (taking into account the maximum number of doses for primary vaccination and, if appropriate, the first revaccination/booster vaccination) and contain the maximum permitted antigen load and, depending on the formulation of the vaccine, the maximum number of vaccine strains.

i) Target and non-target animal safety

The safety of the final product formulation of inactivated vaccines should be assessed in susceptible young sheep that are free of maternally derived antibodies and in pregnant ewes. They should be checked for any local reactions following administration, and, in pregnant ewes, for any effects on the unborn lamb.

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

In the event that a live virus vaccine was developed for BDV, virus seeds that have been passaged at least up to and preferably beyond the passage limit specified for the seed should be inoculated into young lambs to confirm that there is no evidence of disease. If a live attenuated vaccine has been registered for use in pregnant animals, reversion to virulence tests should also include pregnant animals. Live attenuated vaccines should not be transmissible to unvaccinated 'in-contact' animals.

iii) Precautions (hazards)

BDV is not considered to be a human health hazard. Standard good microbiological practice should be adequate for handling the virus in the laboratory. While the inactivated virus in a vaccine should be identified as harmless for people administering the product, adjuvants included in the vaccine may cause injury to people. Manufacturers should provide adequate warnings that medical advice should be sought in the case of self-injection (including for adjuvants, oil-emulsion vaccine, preservatives, etc.) with warnings included on the product label/leaflet so that the vaccinator is aware of any danger.

#### 2.3.3. Efficacy requirements

The potency of the vaccine should be determined by inoculation into seronegative and virus negative lambs, followed by monitoring of the antibody response. Antigen content can be assayed by infectivity titration prior to inactivation and subsequently by ELISA and adjusted as required to a standard level for the particular vaccine. Standardised assay protocols applicable to all vaccines do not exist. Live vaccine batches may be assayed by infectivity titration. Each production batch of vaccine should undergo potency and safety testing as batch release criteria. BVD vaccines must be demonstrated to produce adequate immune responses, as outlined above, when used in their final formulation according to the manufacturer's published instructions.

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

To date, there are no commercially available vaccines for BDV that support use of a true DIVA strategy.

#### 2.3.5. Duration of immunity

Inactivated vaccines are unlikely to provide sustained levels of immunity and it is likely that after an initial course of two or three injections annual booster doses may be required. Insufficient information is available to determine any correlation between vaccinal antibody titres in the dam and fetal protection. As there are likely to be different

commercial formulations and these involve a range of adjuvants, there are likely to be different periods of efficacy. Consequently, duration of immunity data must be generated separately for each commercially available product by undertaking challenge tests at the end of the period for which immunity has been claimed.

#### 2.3.6. Stability

There are no accepted guidelines for the stability of BDV vaccines, but it can be assumed that an inactivated virus vaccine should remain potent for at least 1 year if kept at 4°C and probably longer. Lower temperatures could prolong shelf life but adjuvants in a killed vaccine may preclude this. Bulk antigens that have not been formulated into finished vaccine can be reliably stored frozen at low temperatures, but the antigen quality should be monitored with *in-vitro* assays prior to incorporation into a batch of vaccine.

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611 * *

612 **NB:** At the time of publication (2017) there were no WOAH Reference Laboratories
613 for border disease (please consult the WOAH Web site:
614 https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3http://www.oie.int/).

615 **NB**: FIRST ADOPTED IN 1996. MOST RECENT UPDATES ADOPTED IN 2017.

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1 CHAPTER 3.8.12.

# 2 SHEEP POX AND GOAT POX

## 3 SUMMARY


4 *Sheep pox and goat pox are contagious, viral diseases of sheep and goats characterised by fever,*
5 *generalised papules or nodules, vesicles (rarely), internal lesions (particularly in the lungs), and death. Both*
6 *diseases are caused by strains of capripoxvirus, all of which can infect sheep and goats. Although most of*
7 *the strains examined cause more severe clinical disease in either sheep or goats, some strains have been*
8 *isolated that are equally pathogenic in both species.*

9 *Sheeppox virus (SPPV) and goatpox virus (GTPV) are the causative agents of sheep pox and goat pox, and*
10 *with lumpy skin disease virus (LSDV) make up the genus* Capripoxvirus *in the family* Poxviridae*. Sheep pox*
11 *and goat pox are endemic in Africa north of the Equator, the Middle East and Asia, while some parts of*
12 *Europe have experienced outbreaks recently. See WAHIS (https://wahis.woah.org/#/home) for recent*
13 *information on distribution at the country level. ~~Countries that reported outbreaks of the disease between~~*
14 *~~2010 and 2015 include Bulgaria, Chinese Taipei, Israel, Kazakhstan, Kyrgyzstan, Mongolia, Morocco,~~*
15 *~~Greece and Russia, with Greece, Israel and Russia having experienced recurring incidences.~~*

16 ***Identification of the agent:*** *Laboratory confirmation of capripoxvirus is most rapid using the polymerase*
17 *chain reaction (PCR) method in combination with a clinical history consistent with generalised capripoxvirus*
18 *infection. Isolation of the virus is possible as capripoxviruses will grow on tissue culture of ovine, caprine or*
19 *bovine origin, although field isolates may require up to 14 days to grow or require one or more additional*
20 *tissue culture passage(s). The virus causes intracytoplasmic inclusions that can be clearly seen using*
21 *haematoxylin and eosin staining. The antigen can also be detected in tissue culture using specific sera and*
22 *immunoperoxidase or immunofluorescence techniques. Capripoxvirus antigen and inclusion bodies may be*
23 *seen in stained cryostat or paraffin sections of biopsy or post-mortem lesion material.*

24 *~~An antigen-detection enzyme-linked immunosorbent assay (ELISA) using a polyclonal detection serum~~*
25 *~~raised against a recombinant immunodominant antigen of capripoxvirus has been developed.~~*

26 ***Serological tests:*** *The virus neutralisation test is the most specific serological test. The indirect*
27 *immunofluorescence test is less specific due to cross-reactions with antibody to other poxviruses. Western*
28 *blotting using the reaction between the P32 antigen of capripoxvirus with test sera is both sensitive and*
29 *specific, but is expensive and difficult to carry out. An enzyme-linked immunosorbent assay (ELISA) has*
30 *been developed and validated to detect antibodies to capripoxviruses, however it cannot differentiate*
31 *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.~~*

***Requirements for vaccines:*** *Live and inactivated vaccines have been used for the control of capripoxviruses. All strains of capripoxvirus so far examined share a major neutralisation site and some will cross protect. Inactivated vaccines give, at best, only short-term immunity.*

# A. INTRODUCTION

The *Capripoxvirus* genus, in the family *Poxviridae,* consists of three species – lumpy skin disease virus (LSDV), which causes disease in cattle only (see Chapter 3.4.12), and sheeppox virus (SPPV) and goatpox virus (GT~~P~~PV), which cause sheep pox and goat pox, respectively. Sheep pox and goat pox are characterised by disseminated cutaneous nodules and up to 100% mortality in fully ~~susceptible breeds~~ naïve of sheep and goats. In indigenous animals, generalised disease and mortality are less common, although they are seen where disease has been absent from an area or village for a period of time, when intensive husbandry methods are introduced, or in association with other disease agents, such as peste des petits ruminants virus or foot and mouth disease virus. Sheep pox and goat pox are major constraints to the introduction of exotic breeds of sheep and goats to endemic areas, and to the development of intensive livestock production.

Strains of SPPV and GTPV can pass between sheep and goats, although most cause more severe clinical disease in ~~only one~~ their homologous host species. SPPV and GTPV are transboundary diseases that regularly spread into adjacent, non-endemic areas. Sheep pox and goat pox are endemic in Africa north of the Equator and parts of the Middle East and Asia (see WAHIS for most up-to-date information on distribution: https://wahis.woah.org/#/home). Outbreaks have been reported in non-endemic countries of Asia, Europe and the Middle East.

The incubation period of sheep pox and goat pox is between 8 and 13 days following contact between infected and susceptible animals. It may be as short as 4 days following experimental infection by intradermal inoculation ~~or mechanical transmission by insects~~. Some breeds of European sheep, such as Soay, may die of acute infection before the development of skin lesions. In other breeds there is an initial rise in rectal temperature to above 40°C, followed in 2–5 days by the development of, at first, macules – small circumscribed areas of hyperaemia, which are most obvious on unpigmented skin – and then of papules – hard swellings of between 0.5 and 1 cm in diameter – which may cover the body or be restricted to the groin, axilla and perineum. Papules may be covered by fluid-filled vesicles, but this is rare. Some researchers have distinguished between a vesicular and nodular form of sheep pox and goat pox (Zro *et al*., 2014b).

Within 24 hours of the appearance of generalised papules, affected animals develop rhinitis, conjunctivitis and enlargement of all the superficial lymph nodes, in particular the prescapular lymph nodes. Papules on the eyelids cause blepharitis of varying severity. As the papules on the mucous membranes of the eyes and nose ulcerate, so the discharge becomes mucopurulent, and the mucosae of the mouth, anus, and prepuce or vagina become necrotic. Breathing may become laboured and noisy due to pressure on the upper respiratory tract from the swollen retropharyngeal lymph nodes, due to the developing lung lesions.

If the affected animal does not die in this acute phase of the disease, the papules start to become necrotic from ischaemic necrosis following thrombi formation in the blood vessels at the base of the papule. In the following 5–10 days the papules form scabs, which persist for up to 6 weeks, leaving small scars. The skin lesions are susceptible to fly strike, and secondary pneumonia is common. Anorexia is not usual unless the mouth lesions physically interfere with feeding. Abortion is rare.

On post-mortem examination of the acutely infected animal, the skin lesions are often less obvious than on the live animal. The mucous membranes appear necrotic and all the body lymph nodes are enlarged and oedematous. Papules, which may be ulcerated, can usually be found on the abomasal mucosa, and sometimes on the wall of the rumen and large intestine, on the tongue, hard and soft palate, trachea and oesophagus. Pale areas of approximately 2 cm in diameter may occasionally be seen on the surface of the kidney and liver, and have been reported to be present in the testicles. Numerous hard lesions of up to 5 cm in diameter are commonly observed throughout the lungs, but particularly in the diaphragmatic lobes.

The clinical signs and post-mortem lesions vary considerably with breed of host and strain of capripoxvirus. Indigenous breeds are less susceptible and frequently show only a few lesions, which could be confused with insect bites or contagious pustular dermatitis. However, lambs that have lost their maternally derived immunity, animals that have been kept isolated and animals brought into endemic areas from isolated villages, particularly if they have been subjected to the stress of moving long distances and mixing with other sheep and goats, and their pathogens, can often be seen with generalised and sometimes fatal capripoxvirus infections. Invariably there is high mortality in unprotected imported breeds of sheep and goats following capripoxvirus infection. Surviving animals clear the infection, as there is no evidence of persistently

84 infected animals. Capripoxvirus is not infectious to humans. Capripoxvirus is inactivated at 56°C for 2 hours or 65°C for 30
85 minutes. The virus survives between pH 6.6–8.6. It is susceptible to highly alkaline or acid pH. The virus is sensitive to
86 various chemicals: sodium dodecyl sulphate, ether 20%, chloroform, formalin 1%, sodium 2%, iodine compounds, Virkon
87 2%, quaternary ammonium (0.5%), and phenol 2% for 15 minutes.

88 ## B. DIAGNOSTIC TECHNIQUES

89 ***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 | ++ | ++ | ++ | ++ | ++ | – |
| IFAT | ± | ± | ± | ++ | ± | – |
| IHC | ± | ± | ± | ++ | ± | – |
| PCR | ++ | +++ | ++ | +++ | ++ | – |
| Detection of immune response | | | | | | |
| VNT | ++ | ++ | ++ | ++ | ++ | ++ |
| IFAT | + | + | + | + | + | + |
| ELISA | ++ | ++ | ++ | ++ | ++ | ++ |

90 Key: +++ = recommended for this purpose; ++ recommended but has limitations;
91 + = suitable in very limited circumstances; – = not appropriate for this purpose.
92 IFAT = indirect fluorescent antibody test; IHC = ; immunohistochemistry; PCR = polymerase chain reaction;
93 VNT = virus neutralisation; ELISA = enzyme-linked immunosorbent assay.
94 [(a)]A combination of agent identification methods applied on the same clinical sample is recommended.

95 ### 1. Identification of the agent

96 #### 1.1. Specimen collection and submission

97 Material for virus isolation and antigen detection should be collected by biopsy or at post-mortem from skin
98 papules, lung lesions or lymph nodes. Samples for virus isolation and antigen-detection enzyme-linked
99 immunosorbent assay (ELISA) should be collected within the first week of the occurrence of clinical signs, before
100 the development of neutralising antibodies. Samples for genome detection by polymerase chain reaction (PCR)
101 may be collected before or after the development of neutralising antibody responses. In addition to epithelial
102 lesions, nasal and buccal swabs can be collected because the virus will be present in nasal and saliva
103 discharges. Buffy coat from blood collected into EDTA (ethylene diamine tetra-acetic acid) during the viraemic
104 stage of capripoxvirus infection (before generalisation of lesions or within 4 days of generalisation), can also be
105 used for virus isolation.

106 Samples for histology should include tissue from the surrounding area and should be placed immediately
107 following collection into ten times the sample volume of 10% formalin or neutral buffered 10% formal saline.
108 Tissues in formalin have no special transportation requirements.

Blood samples, for virus isolation from the buffy coat, should be collected in tubes containing anticoagulant, placed immediately on ice and processed as soon as possible. In practice, the blood samples may be kept at 4°C for up to 2 days prior to processing, but should not be frozen or kept at ambient temperatures. Tissues and dry scabs for virus isolation, ~~antigen detection~~ and genome detection should preferably be kept at 4°C, on ice or at –20°C. If it is necessary to transport samples over long distances without refrigeration, the medium should contain 10% glycerol; the samples should be of sufficient size that the transport medium does not penetrate the central part of the biopsy, which should be used for virus isolation/detection.

### 1.2. Virus isolation

Lesion material for virus isolation and genome ~~antigen~~ detection is homogenised. The following is an example of one technique for homogenisation: The tissue is minced using sterile scissors and forceps, and then macerated in a steel ball bearing mixer mill or ground with a sterile pestle in a mortar with sterile sand and an equal volume of sterile phosphate buffered saline (PBS) or serum-free Modified Eagle's Medium (MEM) containing sodium penicillin (1000 international units [IU]/ml), streptomycin sulphate (1 mg/ml), mycostatin (100 IU/ml) or fungizone (2.5 µg/ml) and neomycin (200 IU/ml). The homogenised suspension is freeze–thawed three times and then partially clarified by centrifugation using a bench centrifuge at 600 ***g*** for 10 minutes. In cases where bacterial contamination of the sample is expected (such as when virus is isolated from skin samples), the supernatant can be filtered through a 0.45 µm pore size filter after the centrifugation step, however, the amount of virus in the supernatant might be reduced. Buffy coats may be prepared from 5–8 ml unclotted blood by centrifugation at 600 ***g*** for 15 minutes; the buffy coat is carefully removed into 5 ml of cold double-distilled water using a sterile Pasteur pipette. After 30 seconds, 5 ml of cold double-strength growth medium is added and mixed. The mixture is centrifuged at 600 ***g*** for 15 minutes, the supernatant is discarded and the cell pellet is suspended in 5 ml of growth medium, such as Glasgow's modified Eagle's medium (GMEM). After centrifugation at 600 ***g*** for a further 15 minutes, the resulting pellet is suspended in 5 ml of fresh GMEM. Alternatively, the buffy coat may be separated from a heparinised sample using a density gradient.

Capripoxvirus will grow in tissue culture of bovine, ovine or caprine origin, although primary or secondary cultures of lamb testis (LT) or lamb kidney (LK) cells are considered to be the most susceptible. Care needs to be taken to ensure they are not contaminated with viruses such as bovine viral diarrhoea virus~~, particularly those derived from a wool sheep breed~~ (see chapter 1.1.9). Madin–Darby bovine kidney (MDBK) cells have been shown to be suitable for capripoxvirus isolation (Fay *et al.*, 2020). The following is an example of an isolation technique: either 1 ml of buffy coat cell suspension or 1 ml of clarified biopsy preparation supernatant is inoculated on to a 25 cm$^2$ tissue culture flask of appropriate cells at 90% ~~confluent LT or LK cells~~ confluence, and the supernatant is allowed to adsorb for 1 hour at 37°C. The culture is then washed with warm PBS and covered with 10 ml of a suitable medium, such as GMEM, containing antibiotics and 2% fetal calf serum. If available, tissue culture tubes ~~containing LT or LK cells and a~~, flying cover-slips, or tissue culture microscope slides, ~~are~~ can also infected.

The flasks should be examined daily for 7–14 days for evidence of cytopathic effect (CPE). Contaminated flasks should be discarded. Infected cells develop a characteristic CPE consisting of retraction of the cell membrane from surrounding cells, and eventually rounding of cells and margination of the nuclear chromatin. At first only small areas of CPE can be seen, sometimes as soon as 4 days after infection; over the following 4–6 days these expand to involve the whole cell sheet. If no CPE is apparent by day 7, the culture should be freeze–thawed three times, and clarified supernatant inoculated on to fresh ~~LT or LK~~ cell cultures. At the first sign of CPE in the flasks, or earlier if a number of infected cover-slips are being used, a cover-slip should be removed, fixed in acetone and stained using H&E. Eosinophilic intracytoplasmic inclusion bodies, which are variable in size but up to half the size of the nucleus and surrounded by a clear halo, are indicative of poxvirus infection. Syncytia formation is not a feature of capripoxvirus infection. If the CPE is due to capripoxvirus infection of the cell culture, it can be prevented or delayed by inclusion of specific anti-capripoxvirus serum in the medium; this provides a presumptive identification of the agent. Some strains of capripoxvirus have been adapted to grow on African green monkey kidney (Vero) cells, but these cells are not recommended for primary isolation.

### 1.3. Electron microscopy

The characteristic poxvirus virion can be visualised using a negative-staining preparation technique followed by examination with an electron microscope. There are many different negative-staining protocols, an example is given below:

Material from the original tissue suspension is prepared for transmission electron microscope examination, prior to centrifugation, by floating a 400-mesh hexagon electron microscope grid, with piloform-carbon substrate activated by glow discharge in pentylamine vapour, on to a drop of the suspension placed on parafilm or a wax plate. After 1 minute, the grid is transferred to a drop of Tris/EDTA buffer, pH 7.8, for 20 seconds and then to a drop of 1% phosphotungstic acid, pH 7.2, for 10 seconds. The grid is drained using filter paper, air-dried and

placed in the electron microscope. The capripoxvirus virion is brick shaped, covered in short tubular elements and measures approximately 290 × 270 nm. A host-cell-derived membrane may surround some of the virions, and as many as possible should be examined to confirm their appearance (Kitching & Smale, 1986).

The virions of capripoxvirus are indistinguishable from those of orthopoxvirus, but, apart from *Vaccinia* virus, no orthopoxvirus causes lesions in sheep and goats. However, capripoxvirus is distinguishable from the virions of parapoxvirus, that cause contagious pustular dermatitis, as they are smaller, oval in shape, and each is covered in a single continuous tubular element, which appears as striations over the virion.

### 1.4. Histopathology

Material for histopathology and immunohistochemistry should be prepared by standard techniques (Parvin *et al.,* 2022). Following preparation, and staining with haematoxylin and eosin (H&E), ~~and mounting of the formalin-fixed biopsy material,~~ a number of sections should be examined by light microscopy. On histological examination, the most striking aspects of acute-stage skin lesions are a massive cellular infiltrate, vasculitis and oedema. Early lesions are characterised by marked perivascular cuffing. Initially infiltration is by macrophages, neutrophils and occasionally eosinophils, and as the lesion progresses, by more macrophages, lymphocytes and plasma cells. A characteristic feature of all capripoxvirus infections is the presence of variable numbers of 'sheep pox cells' in the dermis. These sheep pox cells can also occur in other organs where microscopic lesions of sheep and goat pox are present. These cells are large, stellate cells with eosinophilic, poorly defined intracytoplasmic inclusions and vacuolated nuclei. Vasculitis is accompanied by thrombosis and infarction, causing oedema and necrosis. Epidermal changes consist of acanthosis, parakeratosis and hyperkeratosis. Changes in other organs are similar, with a predominant cellular infiltration and vasculitis. Lesions in the upper respiratory tract are characterised by ulceration.

Immunohistochemistry will show capripox virus antigen infiltrated macrophages throughout the subcutis. The capripox virus antigen can occasionally be detected in hair follicle epithelial cells, the endothelium and smooth muscle cells of the blood vessels, and histiocytic cells (Parvin *et al.,* 2022).

### 1.5. Immunological methods

#### 1.5.1. Fluorescent antibody tests

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.

### 1.6. Nucleic acid recognition methods

Amplification methods for detection of ~~the viral DNA genome are specific to the genus~~ *Capripoxvirus* DNA are ~~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.

#### 1.6.1. Conventional PCR methods

Several conventional PCR methods have been reported with varying specificity for capripoxviruses in general, SPPV, or GTPV (Heine *et al.,* 1999; Ireland & Binepal, 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).

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).

216 Test procedure

217 The extraction method described below can be replaced using commercially available DNA extraction
218 kits.

219 i) Freeze and thaw 200 µl of blood in EDTA, semen or tissue culture supernatant and suspend in
220 100 µl of lysis buffer containing 5 M guanidine thiocyanate, 50 mM potassium chloride, 10 mM
221 Tris/HCl (pH 8); and 0.5 ml Tween 20.

222 ii) Cut skin and other tissue samples into fine pieces using a sterile scalpel blade and forceps. Grind
223 with a pestle in a mortar. Suspend the tissue samples in 800 µl of the above mentioned lysis buffer.

224 iii) Add 2 µl of proteinase K (20 mg/ml) to blood samples and 10 µl of proteinase K (20 mg/ml) to tissue
225 samples. Incubate at 56°C for 2 hours or overnight, followed by heating at 100°C for 10 minutes.
226 Add phenol:chloroform:isoamylalcohol (25:24:1 [v/v]) to the samples in a 1:1 ratio. Vortex and
227 incubate at room temperature for 10 minutes. Centrifuge the samples at 16,060 ***g*** for 15 minutes at
228 4°C. Carefully collect the upper, aqueous phase (up to 200 µl) and transfer into a clean 2.0 ml tube.
229 Add two volumes of ice cold ethanol (100%) and 1/10 volume of 3 M sodium acetate (pH 5.3). Place
230 the samples at –20°C for 1 hour. Centrifuge again at 16,060 ***g*** for 15 minutes at 4°C and discard
231 the supernatant. Wash the pellets with ice cold 70% ethanol (100 µl) and centrifuge at 16,060 ***g*** for
232 1 minute at 4°C. Discard the supernatant and dry the pellets thoroughly. Suspend the pellets in
233 30 µl of nuclease-free water and store immediately at –20°C (Tuppurainen *et al.*, 2005).
234 Alternatively a column-based extraction kit may be used.

235 iv) The primers for this PCR assay were developed from the gene encoding the viral attachment
236 protein. The size of the expected amplicon is 192 bp (Ireland & Binepal, 1998). The primers have
237 the following gene sequences:

238 Forward primer 5'-TCC-GAG-CTC-TTT-CCT-GAT-TTT-TCT-TAC-TAT-3'

239 Reverse primer 5'-TAT-GGT-ACC-TAA-ATT-ATA-TAC-GTA-AAT-AAC-3'.

240 v) DNA amplification is carried out in a final volume of 50 µl containing: 5 µl of 10 × PCR buffer, 1.5 µl
241 of $MgCl_2$ (50 mM), 1 µl of dNTP (10 mM), 1 µl of forward primer, 1 µl of reverse primer, 1 µl of DNA
242 template (~10 ng), 0.5 µl of *Taq* DNA polymerase and 39 µl of nuclease-free water. The volume of
243 DNA template required may vary and the volume of nuclease-free water must be adjusted to the
244 final volume of 50 µl.

245 vi) Run the samples in a thermal cycler as follows: 2 minutes at 95°C; then 45 seconds at 95°C,
246 50 seconds at 50°C and 1 minute at 72°C (34 cycles); 2 minutes at 72°C and hold at 4°C until
247 analysis.

248 vii) Mix 10 µl of each sample with loading dye and load onto a 1.5% agarose gel in TAE buffer
249 (Tris/acetate buffer containing EDTA). Load a parallel lane with a 100 bp DNA-marker ladder.
250 Electrophoretically separate the products using approximately 8–10 V/cm for 40–60 minutes and
251 visualise with a suitable DNA stain and transilluminator.

252 **1.6.2. Real-time PCR methods**

253 Several highly sensitive and specific fluorescent detection-based real-time PCR methods have been
254 developed and validated (Balinsky *et al.*, 2008; Bowden *et al.*, 2008; Das *et al.*, 2012; Stubbs *et al.*,
255 2012). Each test detects a small conserved genetic locus within the capripoxvirus genome, but these
256 methods do not discriminate between SPPV, GTPV or LSDV. Real-time PCR methods for direct
257 capripoxvirus ~~genotyping~~ species differentiation without the need for gene sequencing have been
258 described (Haegeman *et al.*, 2013; Gelaye *et al.*, 2013; Lamien *et al.*, 2011b; Wolff *et al.*, 2021).

259 The real-time PCR method described below is a rapid, sensitive and specific method for the detection of
260 the genomic DNA from SPPV, GTPV or LSDV. This assay ~~will~~ is not designed to differentiate ~~between~~
261 the capripoxvirus species.

262 DNA extraction from blood, ~~and~~ tissue and semen

263 A number of DNA extraction kits are commercially available for the ~~isolation~~ extraction of template DNA
264 for real-time PCR. Manufacturer's instructions should always be ~~consulted for guidance on the~~
265 ~~appropriate method for the sample type being extracted~~ followed while using commercial extraction kits.
266 WOAH Reference Laboratories can be contacted for advice on suitable commercial kits.

Real-time PCR

i) The real-time PCR method outlined below uses the primers and probe described by Bowden *et al.* (2008). and further validated by Stubbs *et al.* (2012). Cycling conditions and reagent concentrations can be altered to ensure optimal performance in individual laboratories.

ii) Forward and reverse primers should be prepared at concentrations of 20 µM. A minor grove binder (MGB) TaqMan hydrolysis probe should be prepared at a concentration of 10 µM.

Forward primer: 5'-AAA-ACG-GTA-TAT-GGA-ATA-GAG-TTG-GAA-3'

Reverse primer: 5'-AAA-TGA-AAC-CAA-TGG-ATG-GGA-TA-3'

Probe: 5'-FAM-TGG-CTC-ATA-GAT-TTC-CT-MGB-3'

iii) Mastermix is prepared by combining 10 µl of 2 × real-time PCR mastermix with 0.4 µl of forward primer, 0.4 µl of reverse primer, 0.5 µl of probe and 6.7 µl of RNase free water per reaction.

iv) Add 2 µl of extracted DNA to 18 µl of mastermix in a 96-well PCR plate or PCR strip and perform real-time PCR according to the example given below or similar method:

v) 95°C for 10 minutes followed by 45 cycles of 95°C for 15 seconds, 60°C for 60 seconds. Fluorescence detection should be performed at the end of each cycle.

vi) Following completion of the real-time PCR, a cycle threshold ($C_T$) should be set. Samples with $C_T$ values less than 35 are considered positive. Samples with a $C_T$ value greater than 35 but less than 45 are considered inconclusive and require further investigation. Samples which do not yield a $C_T$ value, i.e. the amplification curve does not cross the threshold, are considered negative.

#### 1.6.3. Isothermal genome amplification

Molecular tests using loop-mediated isothermal amplification (LAMP) to detect capripoxvirus genomes are reported to provide sensitivity and specificity similar to real-time PCR with a simpler method and at lower cost (Das *et al.,* 2012; Murray *et al.,* 2013). Field validation of the Das *et al.* (2012) LAMP method assay has been further reported by (Omoga *et al.,* 2016) and a combination of this universal capripoxvirus test with two additional LAMP assays was reported to show utility in discriminating between to differentiate GTPV and from SPPV (Zhao *et al.,* 2014).

## 2. Serological tests

Detectable levels of antibodies develop 1 week after the animal shows clinical signs. The highest antibody levels are detected within 1–2 months after infection is detected.

### 2.1. Virus neutralisation

A test serum can either be titrated against a constant titre of capripoxvirus (100 $TCID_{50}$ [50% tissue culture infective dose]) or a standard capripoxvirus strain can be titrated against a constant dilution of test serum in order to calculate a neutralisation index. Because of the variable sensitivity of tissue culture to capripoxvirus, and the consequent difficulty of ensuring the use of 100 $TCID_{50}$, the neutralisation index is the preferred method, although it does require a larger volume of test sera. The test is described using 96-well flat-bottomed tissue culture grade microtitre plates, but it can be performed equally well in tissue culture tubes with the appropriate changes to the volumes used, although it is more difficult to read an end-point in tubes. The use of Vero cells in the virus neutralisation test has been reported to give more consistent results (Kitching & Taylor, 1985).

#### 2.1.1. Test procedure

i) Test sera including a negative and a positive control are diluted 1/5 in Eagle's/HEPES (N-2-hydroxyethylpiperazine, N-2-ethanesulphonic acid) and inactivated at 56°C for 30 minutes.

ii) Next, 50 µl of the first inactivated serum is added to columns 1 and 2, rows A to H of the microtitre plate. The second serum is placed in columns 3 and 4, the third in columns 5 and 6, the positive control serum is placed in columns 7 and 8, the negative control serum is placed in columns 9 and 10, and 50 µl of Eagle's/HEPES without serum is placed in columns 11 and 12 and to all wells of row H.

iii) A reference strain of capripoxvirus, usually a vaccine strain known to grow well in tissue culture, with a titre of over $\log_{10}$ 6 $TCID_{50}$ per ml is diluted in Eagle's/HEPES in bijoux bottles to give a log dilution series of $\log_{10}$ 5.0; 4.0; 3.5; 3.0; 2.5; 2.0; 1.5 $TCID_{50}$ per ml (equivalent to $\log_{10}$ 3.7; 2.7; 2.2; 1.7; 1.2; 0.7; 0.2 $TCID_{50}$ per 50 µl).

317 iv) Starting with row G and the most diluted virus preparation, 50 µl of virus is added to each well in that row. This is repeated with each virus dilution, the highest titre virus dilution being placed in row A.

320 v) The plates are covered and incubated for 1 hour at 37°C.

321 vi) ~~LT cells are~~ An appropriate cell suspension (such as MDBK cells) is prepared from pregrown monolayers as a suspension of $10^5$ cells/ml in Eagle's medium containing antibiotics and 2% fetal calf serum. Following incubation of the microtitre plates, 100 µl of cell suspension is added to all the wells, except wells H11 and H12, which serve as control wells for the medium. The remaining wells of row H are cell and serum toxicity controls.

326 vii) The microtitre plates are covered and incubated at 37°C for 9 days.

327 viii) Using an inverted microscope, the monolayers are examined daily starting at day 4 for evidence of CPE. There should be no CPE in the cells of row H. Using the 0240 KSGP vaccine strain of capripoxvirus, the final reading is taken on day 9, and the titre of virus in each duplicate titration is calculated according to the Kärber method. If left longer, there is invariably a 'breakthrough' of virus in which virus that was at first neutralised appears to disassociate from the antibody.

332 ix) *Interpretation of the results:* The neutralisation index is the log titre difference between the titre of the virus in the negative serum and in the test serum. An index of ≥1.5 is positive. The test can be made more sensitive if serum from the same animal is examined before and after infection. Because immunity to capripoxvirus is predominantly cell mediated, a negative result, particularly following vaccination in which the response is necessarily mild, does not imply that the animal from which the serum was taken is not protected.

338 ~~A constant-virus/varying-serum method has been described using serum dilutions in the range 1/5 to 1/500 and fetal calf muscle cells. Because these cells have a lower sensitivity to capripoxvirus than LT cells, the problem of virus 'breakthrough' is overcome.~~

341 **2.2. Indirect fluorescent antibody test**

342 Capripoxvirus-infected tissue culture grown on flying cover-slips or tissue culture microscope slides can be used for the indirect fluorescent antibody test. Uninfected tissue culture control, and positive and negative control sera, should be included in the test. The infected and control cultures are fixed in acetone at –20°C for 10 minutes and stored at 4°C. Dilutions of test sera are made in PBS, starting at 1/5, and positives are identified using an anti-sheep gamma-globulin conjugated with fluorescein isothiocyanate (Davies & Otema, 1978). Cross-reactions can occur with orf, bovine papular stomatitis virus and perhaps other poxviruses.

348 **2.3. Western blot analysis**

349 Western blotting of test sera against capripoxvirus-infected cell lysate provides a sensitive and specific system for the detection of antibody to capripoxvirus structural proteins, although the test is expensive and difficult to carry out (Chand *et al.*, 1994).

352 **2.4. Enzyme-linked immunosorbent assay**

353 ~~No validated ELISA is available for the serological diagnosis of SPP or GTP.~~

354 Both in-house and commercial enzyme-linked immunosorbent assay (ELISAs) are available, but these tests cannot discriminate between antibodies to different capripoxviruses (LSDV or SPPV/GTPV).

356 ## C. REQUIREMENTS FOR VACCINES

357 **[THIS SECTION IS UNDER REVIEW IN THE 2024/2025 REVIEW CYCLE]**

358 ## 1. Background

359 ### 1.1. Rationale and intended use of the product

360 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

363 any other strain (Capstick, 1961). Consequently, it is possible to use a single strain of capripoxvirus to protect
364 both sheep and goats against all field strains of virus, regardless of whether their origin was in Asia or Africa
365 (Kitching *et al.*, 1986; Kitching & Taylor, 1985). However, field evidence suggests some strains are quite host-
366 specific and are used only in sheep against SPPV and only in goat against GTPV.

367 A number of strains of capripoxvirus have had widespread use as live vaccines (Davies & Mbugwa, 1985), for
368 example the Romanian and RM-65 strains used mainly in sheep and the Mysore and Gorgan strains used in
369 goats. The real identity of the commonly used Kenyan sheep and goat pox vaccine virus (KSGP) 0240 was
370 recently shown to be actually LSDV (Tuppurainen *et al.,* 2014). Virus strain identity and attenuation properties
371 must be ascertained and taken into consideration when selecting vaccine strains for use in cattle, sheep and
372 goats. The protective dose depends on the vaccine strain used. Immunity in sheep and goats against
373 capripoxvirus following vaccination with the 0240 strain lasts over a year and the Romanian strain gave
374 protection for at least 30 months.

375 Killed vaccines produced from tissue culture contain only the intracellular mature virion form of the virus, and
376 lack the less robust but biologically crucial extracellular enveloped virion form. As a result, the vaccine does not
377 stimulate immunity against the extracellular enveloped virion, resulting in poor protection. Killed capripoxvirus
378 vaccines provide, at best, only temporary protection.

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

380 General requirements set for the facilities used for the production of vaccines and for the documentation and record keeping
381 throughout the whole manufacturing process are described in Chapter 1.1.8 *Principles of veterinary vaccine production*.
382 The documentation should include the standard operating procedures (SOP) for the method of manufacture and each step
383 for the testing of cells and reagents used in the process, each batches and the final product.

### 384 2.1. Characteristics of the seed

#### 385 2.1.1. Biological characteristics

386 A strain of capripoxvirus used for vaccine production must be accompanied by a history describing its
387 origin and tissue culture or animal passage. It must be safe to use in all breeds of sheep and goats for
388 which it is intended, including pregnant and young animals. It must be non-transmissible, remain
389 attenuated after further tissue culture passage, and provide complete protection against challenge with
390 virulent field strains for a minimum of 1 year. A quantity of master seed vaccine virus should be prepared
391 and stored in order to provide a consistent working seed for regular vaccine production.

#### 392 2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

393 Each master seed must be tested to ensure its identity and shown to be free from adventitious viruses,
394 in particular pestiviruses, such as border disease and bovine viral diarrhoea virus, and free from
395 contamination with bacteria, fungi and/or mycoplasmas. The general procedures for sterility or purity
396 tests are described in chapter 1.1.9. The master seed must also be safe and produce no clinical reaction
397 in all breeds of sheep or goats when given by the recommended route and stimulate complete immunity
398 to capripoxvirus in all breeds of sheep and goats for at least 1 year. The necessary safety and potency
399 tests are described in Section C.2.2.4 *Final product batch tests*.

### 400 2.2. Method of manufacture

401 The method of manufacture should be documented as the Outline of Production.

#### 402 2.2.1. Procedure

403 Vaccine seed should be lyophilised and stored in 2 ml vials at –20°C. It may be stored wet at
404 –20°C, but when wet, is more stable at –70°C or lower. The virus should be cultured in primary or
405 secondary LT or LK cells of wool sheep origin for maximum yield. Vero cells may also be used with
406 suitably adapted strains.

407 Vaccine batches are produced on fresh monolayers of secondary LT or primary LK cells. A vial of seed
408 virus is reconstituted with GMEM or another appropriate medium and inoculated on to an LT or LK
409 monolayer that has been previously washed with warm PBS, and allowed to adsorb for 15 minutes at
410 37°C before being overlaid with additional GMEM. After 4–6 days, there will be extensive (80–90%) CPE.
411 The culture should be examined for any evidence of nonspecific CPE, medium cloudiness or change in
412 medium pH. The culture is freeze–thawed three times, the suspension removed and centrifuged at 600

***g*** for 20 minutes. A second passage may be required to produce sufficient virus for a production batch. Live vaccine may be produced on roller bottles.

The procedure is repeated and the harvests from individually numbered flasks are each mixed separately with an equal volume of sterile and chilled 5% lactalbumin hydrolysate and 10% sucrose, and transferred to individually numbered bottles for storage at –20°C. Prior to storage, 0.2 ml is removed from each bottle for sterility control. An additional 0.2 ml is removed for virus titration; 2 ml pools composed of 0.2 ml samples taken from ten bottles are used. A written record of all the procedures must be kept for all vaccine batches.

Inactivated vaccines are produced, usually from unattenuated field strains of capripoxvirus, grown in tissue culture as described above, inactivated with 0.03% formaldehyde, and mixed with an equal volume of alhydrogel as adjuvant. Formaldehyde is no longer considered to be a suitable inactivant for certain viral vaccines because its mode of action cannot be guaranteed to be totally effective in inactivating all the live virus. This has not been fully investigated for capripoxvirus.

### 2.2.2. Requirements for substrate and media

The specification and source of all ingredients used in the manufacturing procedure should be documented and the freedom from extraneous agents: bacteria, fungi, mycoplasma and any other viruses should be tested. The detailed testing procedure is described in the chapter 1.1.9. The use of antibiotics must meet the requirements of the licensing authority.

### 2.2.3. In-process controls

i) Cells

Cells should be obtained from the testis or kidney of a healthy young lamb from a scrapie-free flock of a wool sheep breed. During cultivation, cells must be observed for any evidence of CPE, and for normal morphology (predominantly fibroblastic). They can usually be passaged successfully up to ten times. When used for vaccine production, uninfected control cultures should be grown in parallel and maintained for at least three additional passages for further observation. They should be checked for the presence of noncytopathic strains of bovine virus diarrhoea or border disease viruses by immunofluorescence or immunoperoxidase techniques. If possible, cells should be prepared and screened prior to vaccine production and stocked in 1–2 ml aliquots containing $2 \times 10^7$ cells/ml in sterile 10% DMSO (dimethyl sulphoxide) and 90% FBS (fetal bovine serum) solution stored in liquid nitrogen.

ii) Serum

Bovine serum used in the growth or maintenance medium must be free from transmissible spongiform encephalopathies (TSEs) and antibody to capripoxvirus, and tested for contamination with pestivirus or any other viruses, extraneous bacteria, mycoplasma or fungi.

iii) Medium

Medium must be tested free from contamination with pestivirus or any other viruses, extraneous bacteria, mycoplasma or fungi.

iv) Virus

Seed virus and final vaccine must be titrated in tissue culture tubes or microtitre plates. Vaccine samples must be examined for the presence of adventitious viruses including cytopathic and noncytopathic strains of pestivirus, and should be mixed with a high-titre capripoxvirus-immune serum that has tested negative for antibody to pestivirus to prevent the vaccine virus itself interfering with the test. The vaccine bulk can be held at –20°C or below until all sterility tests and titrations have been completed, at which time it should be freeze-dried in 1 ml aliquots in vials sufficient for 100 doses. The vaccine harvest diluted with lactalbumin hydrolysate and sucrose should have a minimum titre $\log_{10}$ 4.5 $TCID_{50}$ per ml after freeze-drying, equivalent to a field dose of $\log_{10}$ 2.5 $TCID_{50}$. A further titration is carried out on five randomly chosen vials of the freeze-dried preparation to confirm the titre.

### 2.2.4. Final product batch tests

i) Sterility/purity

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

465 ii) Safety

466–470 The safety studies should be demonstrated by statistically valid vaccination studies using seronegative young sheep and goats of known susceptibility to capripox virus. The procedure described is suitable for vaccine strains such as 0240 that are equally immunogenic in both sheep and goats. The choice of target animal should be adapted for strains with a more restricted host preference.

471 iii) Potency

472–484 Potency tests must be undertaken if the minimum immunising dose of the virus strain 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 wool or hair. $Log_{10}$ 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 should 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 macule/papule is measured at between 8 and 10 days post-challenge. The titre of the challenge virus is calculated for the vaccinated and control animals; a difference of $log_{10}$ titre > 2.5 is taken as evidence of protection.

## 485 2.3. Requirements for authorisation

### 486 2.3.1. Safety requirements

487 i) Target and non-target animal safety

488–490 The vaccine must be safe to use in all breeds of sheep and goats for which it is intended, including young and pregnant animals. It must also be non-transmissible, remain attenuated after further tissue culture passage.

491 Safety tests should be carried out on the final product of each batch as described in Section C.2.2.4.

492–494 The safety of the vaccine in non-target animals must have been demonstrated using mice and guinea-pigs as described in Section C.2.2.4. There should be no evidence of pathology caused by the vaccine.

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

496 The selected final vaccine should not revert to virulence during a further passages in target animals.

497 iii) Environmental consideration

498–501 Attenuated vaccine should not be able to perpetuate autonomously in cattle, sheep or goat populations. Vaccines using the 0240 strain should not be used in *Bos taurus* breeds. Strains of capripoxvirus are not a hazard to human health. There are no precautions other than those described above for sterility and freedom from adventitious agents.

### 502 2.3.2. Efficacy requirements

503 i) For animal production

504–505 The efficacy of the vaccine must be demonstrated in vaccination challenge experiment under laboratory conditions. As described in Section C.2.2.4.

506–508 Once the potency 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.

509 ii) For control and eradication

510–512 Vaccination is the only effective way to control the sheep pox and goat pox outbreaks in endemic countries. Unfortunately, currently no marker vaccines allowing the differentiation of infected from vaccinated animals are available.

Immunity to virulent field virus following vaccination of sheep or goats with the 0240 strain lasts over 1 year, and protection against generalised infection following intradermal challenge lasts at least 3 years and is effective lifelong. The duration of immunity produced by other vaccine strains should be ascertained in both sheep and goats by undertaking controlled trials in an environment in which there is no possibility of field strains of capripoxvirus confusing the results. The inactivated vaccines provide immunity for less than 1 year, and for the reasons given at the beginning of this section, may not give immunity to the form of capripoxvirus usually associated with natural transmission.

#### 2.3.3. Stability

All vaccines are initially given a shelf-life of 24 months before expiry. Real-time stability studies are then conducted to confirm the appropriateness of the expiry date. Multiple batches of the vaccine should be re-titrated periodically throughout the shelf-life to determine the vaccine variability.

Properly freeze-dried preparations of capripox vaccine, particularly those that include a protectant, such as sucrose and lactalbumin hydrolysate, are stable for over 25 years when stored at –20°C and for 2–4 years when stored at 4°C. There is evidence that they are stable at higher temperatures, but no long-term controlled experiments have been reported. The inactivated vaccines must be stored at 4°C, and their shelf- life is usually given as 1 year.

No preservatives other than a protectant, such as sucrose and lactalbumin hydrolysate, are required for the freeze-dried preparation.

## 3. Vaccines based on biotechnology

### 3.1. Vaccines available and their advantages

Currently, no recombinant vaccines for capripoxviruses are commercially available. However, a new generation of capripox vaccines is being developed that uses the capripoxvirus genome as a vector for the genes of other ruminant pathogens such as peste des petits ruminants (PPR) virus (Berhe *et al.*, 2003; Tuppurainen *et al*., 2014).

### 3.2. Special requirements for biotechological vaccines, if any

Not applicable.

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610 * *

611 **NB:** There are WOAH Reference Laboratories for sheep pox and goat pox (please consult the WOAH Web site:
612 https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3http://www.oie.int/).
613 Please contact the WOAH Reference Laboratories for any further information on
614 diagnostic tests, reagents and vaccines for sheep pox and goat pox

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

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1 SECTION 3.9.

2 SUIDAE

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3 CHAPTER 3.9.1.

# 4 AFRICAN SWINE FEVER
# 5 (INFECTION WITH AFRICAN SWINE FEVER VIRUS)

## 6 SUMMARY

7 *African swine fever (ASF) is an infectious disease of domestic and wild pigs of all breeds and ages, caused*
8 *by ASF virus (ASFV). The clinical syndromes vary from peracute, acute, subacute to chronic, depending on*
9 *the virulence of the virus. Acute disease is characterised by high fever, haemorrhages in the*
10 *reticuloendothelial system, and a high mortality rate. Soft ticks of the* Ornithodoros *genus, especially* O.
11 moubata *and* O. erraticus*, have been shown to be both reservoirs and transmission vectors of ASFV. The*
12 *virus is present in tick salivary glands and passed to new hosts (domestic or wild suids) when feeding. It can*
13 *be transmitted sexually between ticks, transovarially to the eggs, or transtadially throughout the tick's life.*

14 *ASFV is the only member of the* Asfarviridae *family, genus* Asfivirus*.*

15 *Laboratory diagnostic procedures for ASF fall into two groups: detection of the virus and serology. The*
16 *selection of the tests to be carried out depends on the disease situation and laboratory diagnostic capacity*
17 *in the area or country.*

18 ***Identification of the agent:*** *Laboratory diagnosis must be directed towards isolation of the virus by*
19 *inoculation of pig leukocyte or bone marrow cultures, the detection of antigen in smears or cryostat sections*
20 *of tissues by fluorescent antibody test and/or the detection of genomic DNA by the polymerase chain reaction*
21 *(PCR) or real-time PCR. The PCRs are excellent, highly sensitive, specific and rapid techniques for ASFV*
22 *detection and are very useful under a wide range of circumstances. They are especially useful if the tissues*
23 *are unsuitable for virus isolation and antigen detection. In doubtful cases, the material is passaged in*
24 *leukocyte cell cultures and the procedures described above are repeated.*

25 ***Serological tests:*** *Pigs that survive natural infection usually develop antibodies against ASFV from 7–10*
26 *days post-infection and these antibodies persist for long periods of time. Where the disease is endemic, or*
27 *where a primary outbreak is caused by a strain of low or moderate virulence, the investigation of new*
28 *outbreaks should include the detection of specific antibodies in serum or extracts of the tissues submitted.*
29 *A variety of methods such as the enzyme-linked immunosorbent assay (ELISA), the indirect fluorescent*

*antibody test (IFAT), the indirect immunoperoxidase test (IPT), and the immunoblotting test (IBT) is available for antibody detection.*

***Requirements for vaccines**: ~~At present, there is no vaccine for ASF.~~ Commercially produced modified live virus vaccines are ~~available and licenced~~ under field evaluation in some countries.*

## A. INTRODUCTION

The current distribution of African swine fever (ASF) extends across more than 50 countries in three continents (Africa, Asia and Europe). Several incursions of ASF out of Africa were reported between the 1960s and 1970s. In 2007, ASF was introduced into Georgia, from where it spread to neighbouring countries including the Russian Federation. From there ASF spread to eastern European countries extending westwards and reaching the European Union in 2014. Further westward and southern spread in Europe has occurred since that time. In all these countries, both hosts – domestic pig and wild boar – were affected by the disease. In August 2018, the People's Republic of China reported its first outbreak of ASF and further spread in Asia has occurred. ASF was identified on the island of Hispaniola (Haiti and the Dominican Republic) in 2021. See WAHIS (https://wahis.woah.org/#/home) for recent information on distribution at the country level.

ASF virus (ASFV) is a complex large, enveloped DNA virus with icosahedral morphology. It is currently classified as the only member of the *Asfaviridae* family, genus *Asfivirus* (Dixon ***et al.***, 2005). More than 60 structural proteins have been identified in intracellular virus particles (200 nm) (Alejo ***et al.,*** 2018). More than a hundred infection-associated proteins have been identified in infected porcine macrophages, and at least 50 of them react with sera from infected or recovered pigs (Sánchez-Vizcaíno & Arias, 2012). The ASFV double-stranded linear DNA genome comprises between 170 and 193 kilobases (kb) and contains between 150 and 167 open reading frames with a conserved central region of about 125 kb and variable ends. These variable regions encode five multigene families that contribute to the variability of the virus genome. The complete genomes of several ASFV strains have been sequenced (Bishop ***et al.,*** 2015; Chapman ***et al.,*** 2011; de Villiers ***et al.,*** 2010; Portugal ***et al.,*** 2015). Different strains of ASFV vary in their ability to cause disease, but at present there is only one recognised serotype of the virus detectable by antibody tests.

The molecular epidemiology of the disease is investigated by sequencing of the 3' terminal end of the B646L open reading frame encoding the p72 protein major capsid protein, which differentiates up to 24 distinct genotypes (Achenbach ***et al.***, 2017; Boshoff ***et al.,*** 2007; Quembo ***et al.*** 2018). To distinguish subgroups among closely related ASFV, sequence analysis of the tandem repeat sequences (TRS), located in the central variable region (CVR) within the B602L gene (Gallardo ***et al.,*** 2009; Lubisi ***et al.,*** 2005; Nix ***et al.,*** 2006) and in the intergenic region between the I73R and I329L genes, at the right end of the genome (Gallardo ***et al.,*** 2014), is undertaken. Several other gene regions such as the E183L encoding p54 protein, the CP204L encoding p30 protein, and the protein encoded by the EP402R gene (CD2v), have been proved as useful tools to analyse ASFVs from different locations and hence track virus spread.

ASF viruses produce a range of syndromes varying from peracute, acute to chronic disease and subclinical infections. Pigs are the only domestic animal species that is naturally infected by ASFV. European wild boar and feral pigs are also susceptible to the disease, exhibiting clinical signs and mortality rates similar to those observed in domestic pigs. In contrast African wild pigs such as warthogs (*Phacochoerus aethiopicus*), bush pigs (*Potamochoerus porcus*) and giant forest hogs (*Hylochoerus meinertzhageni*) are resistant to the disease and show few or no clinical signs. These species of wild pig act as reservoir hosts of ASFV in Africa (Costard ***et al.,*** 2013; Sánchez-Vizcaíno ***et al.,*** 2015).

The incubation period is usually 4–19 days. The more virulent strains produce peracute or acute haemorrhagic disease characterised by high fever, loss of appetite, haemorrhages in the skin and internal organs, and death in 4–10 days, sometimes even before the first clinical signs are observed. Case fatality rates may be as high as 100%. Less virulent strains produce mild clinical signs – slight fever, reduced appetite and depression – which can be readily confused with many other conditions in pigs and may not lead to suspicion of ASF. Moderately virulent strains are recognised that induce variable disease forms, ranging from acute to subacute. Low virulence, non-haemadsorbing strains can produce subclinical non-haemorrhagic infection and seroconversion, but some animals may develop discrete lesions in the lungs or on the skin in areas over bony protrusions and other areas subject to trauma. Animals that have recovered from either acute, subacute or chronic infections may potentially become persistently infected, acting as virus carriers. The biological basis for the persistence of ASFV is still not well understood, nor it is clear what role persistence plays in the epidemiology of the disease.

ASF cannot be differentiated from classical swine fever (CSF) by either clinical or post-mortem examination, and both diseases should be considered in the differential diagnosis of any acute febrile haemorrhagic syndrome of pigs. Bacterial septicaemias may also be confused with ASF and CSF. Laboratory tests are essential to distinguish between these diseases.

In countries free from ASF but suspecting its presence, the laboratory diagnosis must be directed towards isolation of the virus by the inoculation of pig leukocyte or bone marrow cultures, the detection of genomic DNA by polymerase chain reaction (PCR) or the detection of antigen in smears or cryostat sections of tissues by direct fluorescent antibody test (FAT). Currently the PCR is the most sensitive technique and can detect ASFV DNA from a very early stage of infection in tissues, ethylene diamine tetra-acetic acid (EDTA)-blood and serum samples. The PCR is particularly useful if samples submitted are unsuitable for virus isolation and antigen detection because they have undergone putrefaction. Pigs that have recovered from acute, subacute or chronic infections usually exhibit a viraemia for several weeks making the PCR test a very useful tool for the detection of ASFV DNA in pigs infected with low or moderately virulent strains. Virus isolation by the inoculation of pig leukocyte or bone marrow cultures and identification by haemadsorption tests (HAD) are recommended as a confirmatory test when ASF is positive by other methods, particularly in the event of a primary outbreak or a case of ASF.

~~As no vaccine is available, the presence of ASFV antibodies is indicative of previous infection and, as antibodies are produced from the first week of infection and persist for long periods, they are a good marker for the diagnosis of the disease, particularly in subacute and chronic forms.~~

Vaccines should be prepared in accordance with Chapter 1.1.8 *Principles of veterinary vaccine production*. Current ASF modified live virus (MLVs) vaccines are based on the live virus that have been naturally attenuated or attenuated by targeted genetic recombination through cell cultures (Gladue & Borca, 2022). MLV production is based on a seed-lot system consistent with the *European Pharmacopoeia* (11th edition) and that has been validated with respect to virus identity, sterility, purity, potency, stability, safety and immunogenicity (including spread)~~, non-transmissibility, stability and immunogenicity~~. ASF MLV first generation vaccines ~~– defined as those for which peer-reviewed publications are in the public domain –~~ should meet or exceed the minimum standards as described below. ~~Paramount~~ Demonstration of acceptable safety and efficacy against the epidemiologically relevant ASFV field strain(s) circulating in areas where the vaccine is intended for use ~~are~~ is required. At the present time, a variety of mutants (Forth *et al.*, 2023) and recombinants (Zhao *et al.*, 2023) have emerged globally, and the prevalence of these strains is increasing. In addition, there is a risk that vaccine strains will recombine with circulating strains. These conditions should be taken into account in vaccine development. ~~acceptable efficacy should be shown against the B646L (p72) genotype II pandemic virus lineage currently circulating widely in domestic pigs and wild boar.~~

ASF MLV first generation vaccines allowing the differentiation of infected animals from vaccinated animals (DIVA) by suitable methods (e.g. serology-based tests) are preferred. Demonstration of MLV safety and efficacy in pigs at different growth stages (suckling piglets, nursery pigs, fattening pigs), the safety in breeding-age boars, gilts and pregnant sows, ~~and onset and duration of protective immunity,~~ are also preferred ~~but are not required to meet the minimum standard~~. Additional data will likely be required by Regulatory Authorities if these categories are included in the indications for the vaccine. Details of the onset of immunity (the interval of time elapsed between vaccination and challenge if protection is confirmed) and the duration of immunity (the time point at which vaccine-induced immunity begins to decline and provides less protection) are also required to meet minimum standards.

ASF epidemiology is complex with different epidemiological patterns of infection occurring in Africa and Europe. ASF occurs through transmission cycles involving domestic pigs, wild boar, wild African suids, and soft ticks (Sánchez-Vizcaíno *et al.,* 2015). In regions where *Ornithodoros* ~~soft-bodied~~ ticks are present, the detection of ASFV in these reservoirs of infection contributes to a better understanding of the epidemiology of the disease. This is of major importance in establishing effective control and eradication programmes (Costard *et al*., 2013).

ASF is not a zoonotic disease and does not affect public health (Sánchez-Vizcaíno *et al.*, 2009).

ASFV should be handled with an appropriate level of bio-containment, determined by risk analysis in accordance with Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*.

. . .

## C. REQUIREMENTS FOR VACCINES [UNDER REVIEW]

~~At present there is no commercially available vaccine for ASF.~~ Commercially produced modified live virus vaccines are being evaluated and licensed for field use.

## 1. Background

The ASF p72 genotype II strains (ASFV Georgia 2007/1 lineage) (NCBI, 2020) are recognised to be the current highest global threat for domestic pig production worldwide (Penrith *et al.*, 2022). However, genotype I attenuated strains and genotype I/II recombinant strains have been reported to be circulating. In Africa, multiple genotypes are circulating.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of Veterinary Vaccine Production*. Varying additional requirements relating to quality (including purity and potency), safety, and efficacy will apply in particular countries or regions for manufacturers to comply with local regulatory requirements.

Wherever live, virulent ASFV or ASF MLVs are stored, handled and disposed, the appropriate biosecurity level, procedures and practices should be used. The ASF MLV vaccine production facility should meet the requirements for containment outlined in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*.

An optimal ASF MLV first generation vaccine for the target host should have the following general characteristics (minimum standards):

- Safe: demonstrate absence of persistent fever as defined below (see Section 2.3.2) and clinical signs of acute or chronic ASF in vaccinated and in-contact animals, minimal and ideally no vaccine virus transmission, and absence of an increase in virulence (genetic and phenotypic stability);
- Efficacious: protects against mortality, reduces acute disease (fever accompanied by the appearance of clinical signs caused by ASF) and ~~reduces vertical (boar semen and placental) and~~ horizontal disease transmission;
- Quality – purity: free from wild-type ASFV and extraneous microorganisms that could adversely affect the safety, potency or efficacy of the product;
- Quality – ~~potent~~ stability: the ~~$\log_{10}$~~ virus titre maintained throughout the vaccine shelf life that guarantees the efficacy demonstrated by the established minimum immunising (protective) dose.
- ~~Identity~~ Vaccine matching: based on the capacity to protect against the ASFV B646L (p72) genotype II pandemic strain or other p72 genotypes of recognised epidemiologic importance.

Vaccine production should be carried out using a validated, controlled and consistent manufacturing process.

ASF MLV first generation vaccines must be safe (i.e. an acceptable safety profile) for non-target species and the environment in general.

Ideally, ASF MLV first generation vaccines that meet the minimum standards should also fulfil the following additional general characteristics: i) prevents acute and persistent ~~(carrier state)~~ disease; ii) prevents horizontal and vertical disease transmission; iii) induces rapid protective immunity (e.g. < 2 weeks); and iv) confers stable, life-long immunity.

Furthermore, ASF MLV second and future generation vaccines should meet the minimum safety and efficacy standards as ASF MLV first generation vaccines, and ideally provide additional product profile benefits, including but not limited to: i) contain a negative marker allowing the differentiation of infected from vaccinated animals (DIVA) by reliable discriminatory tests such as serology-based tests; and ii) confer broad range of protection against other p72 genotype field strains of varying virulence (low, moderate, and high).

The majority of ASF global vaccine research groups and companies are currently focused on ASF MLV first generation vaccine candidates that are safe and efficacious against ASF viruses belonging to the ASFV p72 genotype II pandemic strain (ASFV Georgia 2007/1 lineage) (NCBI, 2020). More research is needed to determine whether these genotype II-specific MLVs can effectively protect against newly circulating variants of genotype II and recombinant strains.

Currently, two recombinant gene deleted MLV ~~recombinant~~ vaccines (ASFV-G-ΔI177L and ASFV-G-ΔMGF) have been licenced ~~for field use~~ in Vietnam for use in domestic pigs ~~following supervised field testing to evaluate the safety and effectiveness of several vaccine batches~~.

There are numerous, promising ASF MLV vaccine candidates targeting the p72 genotype II pandemic strain under development, including:

- A naturally attenuated field strain (Lv17/WB/Rei1) (Barasona *et al.*, 2019) being developed as an oral bait vaccine for wild boar~~s~~;
- A laboratory thermo-attenuated field strain (ASFV-989) (Bourry *et al.*, 2022);

- Single gene-deleted, recombinant viruses (e.g. SY18ΔI226R, ASFV-G-ΔA137R) (Gladue *et al*., 2021; Zhang *et al*., 2021);
- Double gene-deleted, recombinant viruses (e.g. ASFV-G-Δ9GL/ΔUK; ASFV-SY18-ΔCD2v/UK; Arm-ΔCD2v-ΔA238L) (O'Donnell *et al*., 2016; Pérez-Núñez *et al*., 2022; Teklue *et al*., 2020);
- Multiple gene-deleted, recombinant viruses (ASFV-G-ΔI177L/ΔLVR; ASFV-G-ΔMGF; ~~BA71ΔCD2;~~ HLJ/18-7GD; ASFVGZΔI177LΔCD2vΔMGF) (Borca *et al*., 2021; Chen *et al*., 2020; Kitamura *et al*., 2023; Liu *et al*., 2023; Monteagudo *et al*., 2017; O'Donnell *et al*., 2015).

Information regarding many of these MLV vaccine candidates can be found in a recent review publication (Brake, 2022).

Different DIVA strategies using serological methods (e.g. ELISA) or genome detection methods (e.g. differential real-time PCR) are not widely available for these ASF MLV first generation vaccine candidates. Therefore, there is still room for improvement with respect to marker vaccines and their companion diagnostic tests.

Inactivated (non-replicating) whole virus vaccines are not presently available and may be difficult to develop to meet minimum efficacy standards. Recombinant vectored, subunit vaccine candidates that can be produced in scalable vaccine platform expression systems and mRNA-based ASF vaccines are being evaluated in ongoing laboratory research, testing and evaluation in experimental challenge models. The publicly available *Center of Excellence for African Swine Fever Genomics* (ASFV Genomics, 2022[1]) that provides the structural protein predictions for all 193 ASFV proteins may help accelerate ASF first and second generation vaccine research and development.

Any future use of vaccine candidates should be based on a thorough risk–benefit assessment considering all safety and efficacy features, as well as the potential vaccination scenario. Fit-for-purpose vaccine use scenarios matched to the intended use in a domestic pig-specific type of production system may require different vaccine product profiles or may influence the focus of essential versus ideal vaccine requirements. Prudent use of ASF MLVs as part of strict, controlled vaccination programmes, especially in the areas where ASF is not prevalent, should be implemented.

It is important to know what genotypes of ASFV are circulating in a population before vaccination is introduced. Due to the potential risk of recombination events between circulating low and high virulent field strains with future licensed vaccine strains, and the possibility of reversion to virulence of vaccine strains, strict pharmacovigilance post-vaccination is essential. Field pharmacovigilance data should be collected and analysed during vaccination campaigns using ASF MLV first generation vaccines post-licensing. Active post-vaccination surveillance programmes for the detection of new ASF viruses that may arise from MLV vaccine strains and naturally circulating wild-type virus recombination, as well as revertant vaccine strains, should be implemented. It is also recommended that vaccine manufacturers carry out laboratory experiments to further evaluate the risk of vaccine virus recombination with field and vaccine strains.

As with any ~~MLV~~ vaccine, all ASF ~~MLV~~ vaccines should be used according to the label instructions, under the strict control of the country's Regulatory Authority.

The minimum standards given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national, regional, and veterinary international medicinal product harmonised requirements. Minimum data requirements for an authorisation in exceptional circumstances (e.g. unexpected introduction of the virus, sudden outbreaks of the disease) should be considered where applicable.

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

### 2.1. Characteristics of the seed virus

#### 2.1.1. Biological characteristics of the master seed virus

ASF MLVs are generally produced from ASFV field strains derived from naturally attenuated field isolates or using DNA homologous (genetically targeted) recombination techniques in cell cultures to delete one or more ASFV genes or gene families. These molecular techniques typically involve replacement of the targeted ASFV gene(s) with one or more positive, marker fluorescent (e.g. BFP, eGFP, mCherry) or enzyme-based (e.g. β-glucuronidase) ASFV promoter-reporter gene systems that allow the use of imaging microscopy or flow cytometry to visualise, select, and clone gene-deleted, recombinant, ASF MLVs. MLV production is carried out in cell cultures based on a seed-lot system.

Master seed viruses (MSVs) for MLVs should be selected and produced based on their ease of growth in cell culture, virus yield ($log_{10}$ infectious titre) and genetic stability over multiple cell passages.

[1] http://asfvgenomics.com. Accessed 4/4/2023.

225 Preferably, a continuous well-characterised cell line (e.g. ZMAC-4; PIPEC; IPKM) (Borca *et al.*, 2021; 226 Masujin *et al.*, 2021; Portugal *et al.*, 2020) is used to produce a master cell bank (MCB) on which the 227 MSV and MSV-derived working seed virus (WSV) can be produced. The exact source of the underlying 228 ASFV isolate, the whole genome sequence, and the passage history must be recorded.

229 **2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)**

230 Only MSVs that have been established as sterile, pure (free of wild-type parental virus and free of 231 extraneous agents as described in Chapter 1.1.9 *Tests for sterility and freedom from contamination of* 232 *biological materials intended for veterinary use*, and those listed by the appropriate licensing authorities) 233 and immunogenic, should be used as the vaccine virus (WSV and vaccine batch production). ~~Live~~ 234 ~~vaccines must be shown not to cause disease or other adverse effects in target animals in accordance~~ 235 ~~with chapter 1.1.8, Section 7.1 *Safety tests* (for live attenuated MSVs), that includes target animal safety~~ 236 ~~tests, increase in virulence tests, assessing the risk to the environment) and if possible, no transmission~~ 237 ~~to other animals.~~

238 Identity of the MSV must be confirmed using appropriate methods (e.g. through the use of vaccine strain-239 specific whole genome detection methods such as next generation sequencing).

240 Demonstration of MSV stability over several cell passages is necessary, typically through at least five 241 passages (e.g. MSV+5). For those MLV vaccines for which attenuation is linked to specific characteristics 242 (gene deletion, gene mutations, etc.), genetic stability of attenuation throughout the production process 243 should be confirmed by full genome sequencing and confirmation of the vaccine phenotype, for example, 244 by confirming the virus titre obtained by growth in the cell line used for production ~~using suitable methods.~~ 245 ~~Suitable techniques to demonstrate genetic stability may include but are not limited to: genome~~ 246 ~~sequencing, biochemical, proteomic, genotypic (e.g. detection of genetic markers) and phenotypic strain~~ 247 ~~characterisation~~. If final product yields (infectious titres) are relatively low, as is typically the case with 248 ASFV, demonstration of stability is required for the maximum passage for use in the final product 249 manufacturing as defined by the producer ~~genetic stability at a minimum of MSV+10 should be~~ 250 ~~demonstrated to allow more flexibility in the outline of production. For example, if MSV+8 is the maximum~~ 251 ~~passage for use in final product manufacturing, demonstration of genetic stability to at least MSV+10 is~~ 252 ~~warranted~~.

253 **2.1.3. Validation as a vaccine strain**

254 The vaccine derived from the MSV must be shown to be satisfactory with respect to safety and efficacy.

255 Even if pigs are not known for susceptibility to transmissible spongiform encephalopathy (TSE) agents, 256 consideration should also be given to minimising the risk of TSE transmission by ensuring that animal 257 origin materials from TSE-relevant species, if no alternatives exist for vaccine virus propagation, comply 258 with the measures on minimising the risk of transmission of TSE.

259 Ideally, the vaccine virus in the final product should generally not differ by more than five passages from 260 the master seed lot.

261 ASF vaccines should be presented in a suitable pharmaceutical form (e.g. lyophilisate or liquid form).

262 **2.2. Method of manufacture**

263 **2.2.1. Procedure**

264 The MLV ~~virus~~ is used to infect swine primary cell cultures obtained from specific-pathogen free pigs, the 265 requirements for which are defined in specific monographs (Chapter 2.3.3 *Minimum requirements for the* 266 *organisation and management of a vaccine manufacturing facility*, Section 2.4.2). Compared with primary 267 cell cultures, use of a continuous cell line generally allows for more consistency, higher serial volumes 268 in manufacturing and aligns better with a seed lot system. Thus, preferably a master cell bank based on 269 an established, continuous cell line shown to support genetically stable ASFV replication and acceptable 270 titres over several passages should be used.

271 Cell cultures shall comply with the requirements for cell cultures for production of veterinary vaccines in 272 chapter 1.1.8. Regardless of the production method, the substrate should be harvested under aseptic 273 conditions and may be subjected to appropriate methods to release cell-associated virus (e.g. freeze–274 thaw cycles, detergent lysis). The harvest can be further processed by filtration and other purification 275 methods. A stabiliser or other excipients may be added as appropriate. The vaccine is homogenised to 276 ensure a uniform batch/serial.

### 2.2.2. Requirements for ingredients

All ingredients used for vaccine production should be in line with requirements in chapter 1.1.8.

### 2.2.3. In-process controls

In-process controls will depend on the protocol of production: they include virus titration of bulk antigen and sterility tests.

### 2.2.4. Final product batch tests

i) Sterility

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

ii) Identity

Appropriate methods such as specific genome detection methods (e.g. specific differential real-time PCR) should be used for confirmation of the identity of the vaccine virus and differentiation from the parent strain of the virus as a potential contaminant.

iii) Purity

Appropriate methods should be used to ensure that the final product batch does not contain any residual wild-type ASFV.

iv) Safety

Batch safety testing is to be carried out unless consistent safety of the product is demonstrated and approved in the registration dossier and the production process is approved for consistency in accordance with the standard requirements referred to in chapter 1.1.8.

v) Batch/serial potency

Virus titration is a reliable indicator of vaccine potency once a relationship has been established between the vaccine minimum immunising dose (MID) (minimum protective dose) and titre of the modified live vaccine in vitro. In the absence of a demonstrated correlation between the virus titre and protection, an efficacy test will be necessary (Section C.2.3.3 *Efficacy requirements*, below).

vi) Residual humidity/residual moisture

The test should be carried out consistent with VICH[2] GL26 (*Biologicals: Testing of Residual Moisture*, 2003[3]). Required for MLV vaccines presented as lyophilisates ~~for suspension for injection~~.

## 2.3. Requirements for authorisation/registration/licensing

### 2.3.1. Manufacturing process

For regulatory approval of a vaccine, all relevant details concerning history of the pre-MSV, preparation of MSV, manufacture of the vaccine and quality control testing (Sections C.2.1 *Characteristics of the seed* and C.2.2 *Method of manufacture*) should be submitted to the authorities.

Information shall be provided from three preferably consecutive vaccine batches originating from the same MSV and representative of routine production~~, with a volume not less than 1/10, and more preferably with a volume not less than 1/3 of the typical industrial batch volume~~. The in-process controls are part of the manufacturing process.

### 2.3.2. Safety requirements

For the purpose of gaining regulatory approval, the following safety tests should be performed satisfactorily.

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[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

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.

i) Safety in young animals

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.

The test is conducted using no fewer than eight healthy piglets, and preferably no fewer than ten healthy piglets.

Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

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 [$HAD_{50}$], 50% tissue culture infective dose [$TCID_{50}$]~~, quantitative PCR, etc.~~) (maximum release dose) likely to be contained in one dose of the vaccine.

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.

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).

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).

The vaccine complies with the test if:

- 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;

- ~~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.~~

- 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 exceed 1.5°C and no individual pig should show a rise in temperature above baseline greater than 1.5°C for a period exceeding 3 consecutive days.

- No vaccinated pigs show notable signs of disease by gross pathology

ii) Safety test in pregnant sows and test for transplacental transmission

There is limited ~~currently an absence of~~ published information on ASFV pathogenesis in breeding-age gilts and in pregnant sows associated with ASFV transplacental infection and fetus abortion/stillbirth. If a label claim is pursued for use in breeding age gilts and sows, then a safety

363 study in line with VICH GL44 (*Guidelines on Target Animal Safety for Veterinary Live and*
364 *Inactivated Vaccines, Section 2.2. Reproductive Safety Test*, 2009[4]) should be completed.

365 iii) Horizontal transmission

366 The test is conducted using no fewer than 12 healthy piglets, a minimum of 6 4-weeks old and not
367 older than 10-weeks old and of the same origin, that do not have antibodies against ASFV, and
368 blood samples are negative on real-time PCR. All piglets are housed together from day 0 and the
369 number of vaccinated animals is the same as the number of naïve, contact animals. Co-mingle
370 equal numbers of vaccinated and naïve, contact piglets from day 0 in the same pen or room.

371 Use vaccine virus at the least attenuated passage level that will be present between the master
372 seed lot and a batch of the vaccine. Administer by each recommended route of administration to
373 no fewer than six piglets a quantity of the vaccine virus equivalent to not less than the maximum
374 virus titre (maximum release dose) likely to be contained in 1 dose of the vaccine.

375 To obtain individual and group mean baseline temperatures, the body temperature of each naïve,
376 contact piglet is measured on at least the 3 consecutive days preceding co-mingling with vaccinated
377 piglets. The body temperature of each naïve, contact piglet is then measured daily for at least 45
378 days, preferably 60 days.

379 To confirm the presence or absence of fever accompanied by disease, observe the naïve, contact
380 piglets daily for at least 45 days, preferably 60 days. On each day during the observation period the
381 maximum increase in body temperature above the baseline observed for each pig will be used to
382 calculate the daily group mean temperature rise. This mean value should not exceed 1.5°C and no
383 individual pig should show a rise in temperature above baseline greater than 1.5°C for a period
384 exceeding 3 consecutive days. Carry out the daily observations for signs of acute and chronic
385 clinical disease using a quantitative clinical scoring system adding the values for multiple clinical
386 signs (e.g. Gallardo *et al.*, 2015a). These clinical signs should include fever, anorexia, recumbency,
387 skin haemorrhage or cyanosis, joint swelling and necrotic lesions around the joints, respiratory
388 distress and digestive findings.

389 In addition, Blood should be taken from the naïve contact piglets at least twice a week for the first
390 21 days post-vaccination and then on a weekly basis. From the blood samples, determine vaccine
391 virus titres by quantitative virus isolation ($HAD_{50}$/ml, $TCID_{50}$/ml or other methods, e.g. titration using
392 IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should
393 be confirmed by infectious virus titration as described above infectious virus titres by quantitative
394 virus isolation (e.g. $HAD_{50}$/ml or $TCID_{50}$/ml) and using a real-time PCR test.

395 If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR
396 test only may be used.

397 Collect blood (serum) samples from the naïve contact pigs at least at day 21 and day 28 days and
398 carry out an appropriate test to detect vaccine virus antibodies. At a minimum of 45 days, humanely
399 euthanise all naïve, contact piglets. Conduct gross pathology on spleen, lung, tonsil, and kidney
400 tissue samples and at least three different lymph nodes. Determine virus titres in all collected
401 samples by quantitative virus isolation ($HAD_{50}$/mg or $TCID_{50}$/mg) or other appropriate methods (e.g.
402 IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should
403 be confirmed by infectious virus titration as described above and real-time(RT)-PCR (see Section
404 B.1. Identification of the agent). If the vaccine virus is non-haemadsorbing or does not cause
405 cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT
406 detection) may be used.

407 The vaccine complies with the test if:

408 • No vaccinated or naïve contact piglet shows abnormal (local or systemic) reactions or notable
409 signs of disease, reaches the predetermined humane endpoint defined in the clinical scoring
410 system or dies from causes attributable to the vaccine;

411 • On each day during the observation period the maximum increase in body temperature above
412 the baseline observed for each pig will be used to calculate the daily group mean temperature
413 rise. This mean value should not exceed 1.5°C and no individual pig should show a rise in

4 https://www.ema.europa.eu/en/documents/scientific-guideline/vich-gl44-target-animal-safety-veterinary-live-inactived-vaccines-step-7_en.pdf.

414 temperature above baseline greater than 1.5°C for a period exceeding 3 consecutive days
415 The average body temperature increase for all naïve, contact piglets (group mean) for the
416 observation period does not exceed 1.5°C: above baseline; and no individual piglet shows a
417 temperature rise above baseline greater than 2.5°C for a period exceeding 3 days;

418 • No naïve, contact piglet shows notable signs of disease by gross pathology and no virus is
419 detected in their blood or tissue samples;

420 • No or a low percentage of contact piglets test both real-time PCR positive and seropositive
421 No naïve contact pigs test positive for antibodies to the vaccine virus.

422 iv) Post-vaccination kinetics of viral replication (MLV blood and tissue dissemination) study

423 Prior to the reversion to virulence study (Section C2.3.2.v. below), a minimum of one study should
424 be performed to determine the post-vaccination kinetics of virus replication in the blood (viremia),
425 tissues and viral shedding.

426 The test consists of the administration of the vaccine virus from the master seed lot to no fewer
427 than eight healthy piglets, and preferably ten healthy piglets, a minimum of 6 4-weeks old and not
428 older than 10-weeks old and of the same origin, that do not have antibodies against ASFV, and
429 blood samples are negative on real-time PCR.

430 Administer to each piglet, using the recommended route of administration most likely to result in
431 spread (such as the intramuscular route or intranasal route), a quantity of the master seed vaccine
432 virus equivalent to not less than the maximum virus titre (maximum release dose) likely to be
433 contained in 1 dose of the final product of the vaccine.

434 Record daily body temperatures and observe inoculated animals daily for clinical disease for at
435 least 45 days, preferably 60 days.

436 Carry out the daily observations for signs of acute and chronic clinical disease using a quantitative
437 clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.* (2015a).
438 These clinical signs should include fever, anorexia, recumbency, skin haemorrhage or cyanosis,
439 joint swelling and necrotic lesions around the joints, respiratory distress and digestive findings.

440 Collect blood samples from all the piglets at least two times per week from 3 days post-vaccination
441 for the first 2 weeks, then weekly for the duration of the test. Determine vaccine virus titres by
442 quantitative virus isolation ($HAD_{50}$/ml or $TCID_{50}$/ml) or other appropriate methods (e.g. titration using
443 IPT or FAT detection). Quantitative PCR may be used to detect positive samples but results should
444 be confirmed by infectious virus titration as described above and using a real-time PCR test. If the
445 vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test only
446 may be used.

447 Determine which blood timepoint(s) should be used in the design of the reversion to virulence study
448 (Section C2.3.2.v. below), for example, specific blood sample(s) at specific timepoints that show
449 the highest titres should be considered for selection and use in the reversion to virulence study.

450 Collect oral, nasal and faecal swab samples (preferably devoid of blood to minimise assay
451 interference) at least two times per week from 3-days post-vaccination for the first 2 weeks, then
452 weekly for the duration of the test. Test the swabs for the presence of vaccine virus. Determine
453 virus titres in all collected samples by quantitative virus isolation ($HAD_{50}$/ml or $TCID_{50}$/ml) or other
454 appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to
455 detect positive samples, but results should be confirmed by infectious virus titration as described
456 above and using a real-time PCR test. If the vaccine virus is non-haemadsorbing or does not cause
457 cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT
458 detection) may be used.

459 Euthanise at least two piglets on days 5, 7, 14, 21, and preferably on day 28 (±2 days at each
460 timepoint) and collect spleen, lung, tonsil, kidney tissue samples and at least three different lymph
461 nodes (which should include lymph node closest to site of inoculation, gastrohepatic and
462 submandibular nodes). Determine virus titres in all collected samples by quantitative virus isolation
463 ($HAD_{50}$/mg or $TCID_{50}$/mg) or other appropriate methods (e.g. titration using IPT or FAT detection).
464 Quantitative PCR may be used to detect positive samples, but results should be confirmed by
465 infectious virus titration as described above and using real-time PCR test. If the vaccine virus is

466 ~~non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate~~
467 ~~method (e.g. titration using IPT or FAT detection) may be used~~.

468 Determine which tissue(s) and timepoint(s) should be used to aid in the design of the reversion to
469 virulence study (Section C.2.3.2.v), for example, specific tissues at specific timepoints which show
470 the highest titres should be considered for selection and use in the reversion to virulence study.

v) Reversion to virulence

The test carried out should be consistent with VICH GL41 (Examination of live veterinary vaccines in target animals for absence of reversion to virulence, 2008[5]).

The test for increase in virulence consists of the administration of the vaccine master seed virus to healthy piglets of an age (e.g. between ~~6~~ 4 weeks and 10 weeks old) suitable for recovery of the strain and of the same origin, that do not have antibodies against ASFV, and blood samples that are negative on real-time PCR. This protocol is typically repeated five times.

*First passage (p1)*

Administer to no fewer than two piglets, and preferably no fewer than four piglets using the intended route of administration for the final product, a quantity of the master seed vaccine virus equivalent to not less than the maximum virus titre (maximum release dose) likely to be contained in 1 dose of the final product of the vaccine. Observe inoculated animals daily for the appearance of ~~at least two and preferably at least three~~ clinical signs ~~and record daily body temperatures~~ using a quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.*, 2015a) and record daily body temperatures.

Based on results from at least one completed post-vaccination kinetics of viral replication (MLV ~~vaccine shed and spread (virus~~ blood and tissue dissemination study (Section C.2.3.2.iv above), collect an appropriate quantity of blood from each piglet on the predetermined ~~single~~ timepoint(s) (day ~~5~~ 3–13). Determine virus titres in individual blood samples by quantitative virus isolation (HAD$_{50}$/ml or TCID$_{50}$/ml) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above ~~and by real-time PCR. If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT detection) may be used~~. Identify the individual blood sample(s) with the highest infectious titre and reserve for the subsequent *in-vivo* passage (second pass, p2).

Based on results from at least one completed ~~vaccine virus~~ MLV blood and tissue ~~distribution~~ dissemination study (Section C.2.3.2.iv above), euthanise piglets on the predetermined timepoint (i.e. day 5, 7, 14, 21, or 28). Determine infectious virus titres in individual tissue samples by quantitative virus isolation (HAD$_{50}$/ml or TCID$_{50}$/ml) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above. ~~If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT detection) may be used.~~ Identify individual tissue-sample type(s) with the highest infectious titre. Pool the tissues with the highest titres from different organs from ~~all~~ each animal~~s~~ ~~with the highest titres~~ and prepare ~~at least~~ a ~~10%~~ virus suspension to obtain a virus titre within the range used for inoculation in PBS, pH 7.2 kept at 4°C or at –70°C for longer storage.

Test each blood and tissue sample pool used for inoculation by PCR to confirm the absence of potential viral agent contaminants (i.e. CSFV, FMDV, PRRS, PCV2). Blood and pooled tissue (p1) are used to inoculate 2 ml of positive material diluted to the maximum release dose likely to be contained in 1 dose of the vaccine using the intended route of administration for the final product to each of at least two and ideally at least four further pigs of the same age and origin.

~~*Second pass (p2)*~~

If no virus is found at passage 1 (p1), repeat the administration by the intended route ~~once~~ again with the same pooled material (blood and pooled tissue, p1) in another ten healthy piglets of the same age and origin. If no virus is found at this point ~~during this second passage (p2) at this point~~, end the process here.

*Second passage (p2)*

If ~~however~~ virus is found in p1, carry out a second series of passages by administering 2 ml of positive material diluted to the maximum release dose likely to be contained in 1 dose of the vaccine

[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.

using the intended route of administration for the final product to each of no fewer than two piglets, and preferably no fewer than four piglets of the same age and origin. Observe inoculated animals daily for the appearance of ~~at least two and preferably at least three~~ clinical signs using a quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.*, 2015a), ~~and~~ record daily body temperatures and determine infectious virus titres in individual blood and tissue samples as described for p1 above.

~~*Third and fourth pass (p3 and p4)*~~

If no virus is found at ~~in~~ (p2), repeat the ~~intramuscular~~ administration by the intended route ~~once again~~ with the same pooled material (blood and pooled tissue, p2) in another eight healthy piglets of the same age and origin. If no virus is found at this point, end the process here.

*Third and fourth passage (p3 and p4)*

If~~, however,~~ virus is found on p2, carry out this passage operation no fewer than two additional times (p3 and p4) (to each of no fewer than two piglets, and preferably no fewer than four piglets of the same age and origin) and verifying the presence of the virus at each passage in blood and tissues. Observe inoculated animals daily for the appearance of ~~at least two and preferably at least three~~ clinical signs using a quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.*, 2015a) and record daily body temperatures.

*Fifth passage (p5)*

Administer 2 ml of the blood and pooled tissue (p4) to each of at least eight healthy piglets of the same age and origin. Observe inoculated animals daily for at least 28 days post-inoculation for the appearance of ~~at least two and preferably at least three~~ clinical signs using a quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.*, 2015a), ~~and~~ record daily body temperature and determine infectious virus titres in individual blood and tissue samples as described above.

The vaccine virus complies with the test if:

- 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; and
- There is no indication of increasing virulence (as monitored by daily body temperature accompanied by clinical sign observations) of the maximally passaged virus compared with the master seed virus.

At a minimum, a safe MLV vaccine shall demonstrate ALL the following features (minimal standards):

- Absence of fever (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 exceed 1.5°C and no individual pig should show a rise in temperature above baseline greater than 1.5°C ~~(defined as 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);
- Absence of chronic and acute clinical signs and gross pathology over the entire test period or ~~minimal chronic~~ mild clinical signs (~~defined as~~ e.g. mild swollen joints with a low clinical score that resolve within 1 week).
- Minimal (defined as no naïve, contact piglet shows notable signs of disease by clinical signs and gross pathology and no or a low percentage of contact piglets test both real-time PCR positive and seropositive) or no vaccine virus transmission (defined as no naïve, contact piglet shows notable signs of disease by clinical signs and gross pathology and no contact piglets test both real-time PCR positive and seropositive) over the entire test period;
- Absence of an increase in virulence (genetic and phenotypic stability) (complies with the reversion to virulence test).

In addition, for regulatory approval, ASF MLV ~~the~~ vaccines ~~in their commercial presentation before being authorised for general use~~ should be tested for safety ~~in the~~ under field conditions (see

chapter 1.1.8 Section 7.2.3). ~~Additional~~ Field safety studies generally ~~evaluation studies~~ may include measurement of body temperatures, observation of local or systemic reactions and, where appropriate, performance measurements ~~but are not limited to: environmental persistence (e.g. determination of virus recovery from bedding or other surfaces), assessment of immunosuppression, and negative impacts on performance~~.

### 2.3.3. Efficacy requirements

i) Protective dose

Vaccine efficacy is estimated in immunised animals directly, by evaluating their resistance to live virus challenge. The test consists of a vaccination/challenge trial in piglets a minimum of ~~6~~ 4-weeks old and not more than 10-weeks old, free of antibodies to ASFV, and negative blood samples by real-time PCR. The test is conducted using no fewer than 15 and preferably no fewer than 24 vaccinated pigs, and no fewer than five non-vaccinated control piglets.

The test is conducted to determine the minimal immunising dose (MID) (also referred to as the minimal protective dose [MPD] or protective fraction); using at least three groups of no fewer than five and preferably not fewer than eight vaccinated piglets per group, and one additional group of no fewer than five non-vaccinated piglets of the same age and origin as controls. Use vaccine containing virus at the highest passage level that will be present in a batch of vaccine.

Each group of piglets, except the control group, is immunised with a different vaccine virus content in the same vaccine volume. In at least one vaccinated group, piglets are immunised with a vaccine dose containing not more than the minimum virus titre (minimum release dose) likely to be contained in one dose of the vaccine as stated on the label.

Twenty-eight days (±2 days) after the single ~~injection~~ dose of vaccine (or if using two ~~injections~~ doses of the vaccine then 28 days [±2 days] following the second ~~injection~~ dose), challenge all the piglets by the intramuscular route. If previous studies have demonstrated acceptable efficacy using IM challenge, then a different challenge route (e.g. direct contact, oral or oronasal) may be used. Challenged, vaccinated piglets may be housed in one or more separate pens in the same room or in different rooms. Challenged, naïve controls can be housed in one or more rooms that are separate from challenged, vaccinated piglets.

Carry out the test using an ASFV representative strain of the epidemiologically relevant field strain(s) where the vaccine is intended for use (e.g. ASFV B646L [p72] genotype II pandemic strain and other p72 virulent genotype of recognised epidemiologic importance). For gene deleted, recombinant MLV viruses, if neither challenge virus type is available, then carry out the test with the parental, virulent virus used to generate the MLV recombinant virus. Use a 10e3–10e4 $HAD_{50}$ (or $TCID_{50}$ for non-HAD viruses) challenge dose sufficient to cause death or meet the humane endpoint in 100% of the nonvaccinated piglets in less than 21 days. Higher or lower challenge doses can be considered if appropriately justified.

The rectal temperature of each vaccinated piglet is measured on at least the 3 days preceding administration of the challenge virus, at the time of challenge, 4 hours after challenge, and then daily for the observation period of at least ~~28~~ 45 days, preferably ~~35~~ 60 days. ~~Observe the piglets at least daily for at least 28 days, preferably 35 days.~~ Carry out the daily observations for signs of acute and chronic clinical disease using a quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.*, 2015). 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.

Collect oral, nasal, anal and blood samples from the vaccinated challenged piglets at least two times ~~once~~ per week from 3 days post-challenge for at least ~~28~~ 14 days, then weekly up to 35 days post-challenge and then every 14 days up to the end of the observation period ~~preferably 35 days~~. From the blood samples, determine infectious virus titres by quantitative virus isolation ($HAD_{50}$/ml or $TCID_{50}$/ml) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above ~~and using a real-time PCR test. If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test only may be used~~.

At the end of the test period, humanely euthanise all vaccinated challenged piglets. Conduct gross pathology (and histopathology if considered necessary) on spleen, lung, tonsil, and kidney tissue samples and at least three different lymph nodes (which should include lymph node closest to site

629 of inoculation, gastrohepatic and submandibular nodes). Determine virus titres in all collected
630 samples by quantitative virus isolation ($HAD_{50}$/mg or $TCID_{50}$/mg) or other appropriate methods (e.g.
631 titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but
632 results should be confirmed by infectious virus titration as described above and real-time PCR (see
633 Section B.1. Identification of the agent). If the vaccine virus is non-haemadsorbing or does not
634 cause cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT
635 or FAT detection) may be used.

636 The test is invalid if fewer than 100% the difference between in the number of unvaccinated control
637 piglets infected with the live challenge virus and the number of vaccinated / challenged piglets
638 vaccinated with the minimum release dose that die or reach a humane endpoint is not statistically
639 significant.

640 The vaccine (or a specific vaccine virus dose if conducting a vaccine dose titration study) complies
641 with the test if:

642 • No vaccinated challenged piglet dies or shows abnormal (local or systemic) reactions, reaches
643 the humane endpoint or dies from causes attributable to ASF;

644 • On each day during the observation period the maximum increase in body temperature above
645 the baseline observed for each pig will be used to calculate the daily group mean. This mean
646 value should not exceed 1.5°C and no individual pig should show a rise in temperature above
647 baseline greater than 2.0°C for a period exceeding 2 consecutive days The average body
648 temperature increase for all vaccinated challenged piglets (group mean) for the observation
649 period does not exceed 2.0°C above baseline; and no individual piglet shows a temperature
650 rise above baseline greater than 2.0°C;

651 • The vaccinated challenged piglets display a reduction or absence of typical acute clinical signs
652 of disease and gross pathology and a reduction or absence of challenge virus levels in blood
653 and tissues.

654 ii) Assessment for horizontal transmission (challenge virus shed and spread study)

655 The ASF basic reproduction number, R0, can be defined as the average number of secondary ASF
656 disease cases caused by a single ASFV infectious pig during its entire infectious period in a fully
657 susceptible population (Hayes *et al*., 2021). In general, if the ASFV effective reproduction number
658 Re=R0 × (S/N) (S= susceptible pigs; N= total number of pigs in a given population) is greater than
659 1.0, disease is predicted to spread. Ideally, ASF vaccination should reduce Re to less than 1.0 by
660 reducing the number of susceptible, naïve, contact pigs exposed to vaccinated, infected pigs.

661 To evaluate ASF vaccine impact on ASF disease transmission, the test consists of a
662 vaccination/challenge trial in piglets a minimum of 6 4-weeks old and not older than 10-weeks old,
663 free of antibodies to ASFV, and negative blood samples by real-time PCR.

664 The test is conducted using no fewer than 15 healthy piglets at a ratio comprising twice the number
665 of vaccinated piglets to naïve piglets (e.g. ten vaccinated and five naïve). Use vaccine containing
666 virus at the highest passage level that will be present in a batch of the vaccine.

667 The quantity of vaccine virus administered to each pig is equivalent to be not less than the minimum
668 virus titre (minimum dose) likely to be contained in one dose of the vaccine as stated on the label.
669 Following immunisation, vaccinated and naïve piglets should continue to be co-mingled.

670 Twenty-eight days (±2 days) after the single injection dose of vaccine (or if using two injections
671 doses of the vaccine then 28 days [±2 days] following the second injection dose), temporarily
672 separate [into different pen(s) or room(s)] all vaccinated piglets from naïve piglets. Challenge all
673 vaccinated piglets by the intramuscular or other previously verified route. Carry out the challenge
674 using an ASFV representative strain of the epidemiologically relevant field strain(s) where the
675 vaccine is intended for use (e.g. ASFV B646L [p72] genotype II pandemic strain and other p72
676 virulent genotype of recognised epidemiological importance). For gene deleted, recombinant MLV
677 viruses, if neither challenge virus type is available, then carry out the test with the parental, virulent
678 virus used to generate the MLV recombinant virus. Use a 10e3–10e4 $HAD_{50}$ (or $TCID_{50}$ for non-
679 HAD viruses challenge dose sufficient to cause death or met the humane endpoint in 100% of the
680 nonvaccinated piglets in less than 21 days. Higher or lower challenge doses can be considered if
681 appropriately justified.

Approximately 18–24 hours later, re-introduce naïve piglets to vaccinated, challenged piglets and allow for direct nose to nose contact exposure with vaccinated, challenged piglets. Allow for continuous contact exposure by co-mingling both groups through the end of the study. If more than one pen or room is used for co-housing, following reintroduction initially maintain a ratio of 2:1 of challenged, vaccinated piglets to contact exposed, naïve piglets.

The rectal temperature of each contact piglet is measured on at least the 3 days preceding administration of the challenge virus to vaccinated pigs, immediately prior to direct contact exposure, 4 hours post-contact exposure, and then daily for at least 28~~, preferably 35~~ days and twice a week for at least 60 days. Observe all contact exposed piglets ~~at least~~ daily for at least 28 days, and then twice a week for at least 60 days ~~preferably for at least 35 days~~.

Carry out the ~~daily~~ observations in each contact piglet for signs of acute and chronic clinical 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.

In addition, ~~blood should be taken from the naïve contact piglets at least twice a week from 3 days post-contact exposure for the duration~~ collect blood samples from the contact piglets at least two times per week from 3 days post-contact for at least 14 days, then weekly up to 35 days post-contact exposure and then every 14 days up to the end of the test period. Determine virus titres in all collected samples by quantitative virus isolation (HAD$_{50}$/mg or TCID$_{50}$/mg) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above ~~From the blood samples, determine infectious challenge virus titres by quantitative virus isolation (HAD$_{50}$/ml or TCID$_{50}$/ml) and using a real-time PCR test. If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test only may be used~~.

Collect blood (serum) samples from the naïve contact pigs at least at day 21 and day 28 (±2 days), and at the end of the test period, and carry out an appropriate test to detect vaccine virus antibodies.

Collect oral, nasal and faecal swab samples (preferably devoid of blood to minimise assay interference) from all contact-exposed naïve piglets at least two times per week from 3-days post-contact exposure for the first 2 weeks, then weekly for the duration of the test and test swabs for the presence of challenge virus. Determine virus titres in all collected samples by quantitative virus isolation (HAD$_{50}$/mg or TCID$_{50}$/mg) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above ~~Determine virus titres in all collected samples by quantitative virus isolation (HAD$_{50}$/ml or TCID$_{50}$/ml) and using a real-time PCR test. If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT detection) may be used~~.

At the end of the test period, humanely euthanise all contact 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). Determine virus titres in all collected samples by quantitative virus isolation (HAD$_{50}$/mg or TCID$_{50}$/mg) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above ~~Determine virus titres in all collected samples by quantitative virus isolation (HAD$_{50}$/mg or TCID$_{50}$/mg) and real-time PCR (see Section B.1. Identification of the agent). If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT detection) may be used~~.

The test is invalid if the vaccine fails to comply with the compliance criteria described for the protected dose test in vaccinated pigs (Section C.2.3.3.i above).

If the manufacturer claims that the vaccine induces sterilising immunity, the vaccine complies with the test for a reduction in horizontal disease transmission if all the following conditions are satisfied:

- No naïve, contact exposed piglet shows abnormal (local or systemic) reactions, reaches the defined humane endpoint or dies from causes attributable to ASF;
- No naïve, contact exposed piglet displays fever accompanied by typical signs of disease, including gross pathology.

- Naïve contact pigs show an absence of challenge virus in blood and tissues.
- No naïve contact pigs test positive for antibodies to the challenge virus.

Otherwise, the vaccine complies with the test for a reduction in horizontal disease transmission if:

- Naïve contact pigs show a reduction or absence of challenge virus levels in blood and tissues.
- None of or a reduced number of naïve contact exposed pigs test positive for antibodies to the challenge virus.

At a minimum, an efficacious MLV vaccine shall demonstrate ALL the following features (minimal standards):

- Protects against mortality;
- Reduces acute disease (fever accompanied by a reduction of typical clinical and pathological signs of acute disease)
- Reduces levels of viral shedding and viraemia.
- Reduces horizontal disease transmission (~~no~~ none of or a reduced number of naïve, contact exposed piglets shows abnormal [local or systemic] reactions, reaches the humane endpoint or dies from causes attributable to ASF, ~~and~~ displays fever accompanied by typical acute disease signs caused by ASF) and test positive for antibodies to the challenge virus.
- ~~Reduces levels of viral shedding and viraemia.~~

In general, for regulatory approval, ASF MLV ~~addition, the~~ vaccines ~~in their commercial presentation before being authorised for general use~~ should be tested for efficacy ~~in the~~ under field conditions (see chapter 1.1.8 Section 7.2.3). ~~Additional~~ Field efficacy ~~evaluation~~ studies ~~may~~ generally include ~~but are not limited to: onset of immunity, duration of immunity, and impact on disease transmission~~ measurement of relevant efficacy parameters including but limited to mortality, clinical signs, impact on disease transmission, performance parameters.

### 2.3.4. Duration of immunity

Although not included in the guidance for ASF MLV first generation vaccines, manufacturers are ~~encouraged~~ required, as part of the authorisation procedure, to define and demonstrate the duration of immunity of a given vaccine ~~by evaluation of potency at the end of the claimed period of protection~~.

### 2.3.5. Stability

Stability of the vaccine should be demonstrated over the shelf life recommended for the product. Although not included in the standards for first generation MLV ASF vaccines, manufacturers are ~~encouraged~~ required, as part of the authorisation procedure, to generate data supporting the retention of immunogenicity over a defined period of ~~validity~~ time of a lyophilised or other pharmaceutical form of the ASF vaccine as part of the authorisation procedure.

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937 *

938 * *

939 **NB:** There are WOAH Reference Laboratories for African swine fever
940 (please consult the WOAH Web site:
941 https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
942 Please contact the WOAH Reference Laboratories for any further information on
943 diagnostic tests and reagents for African swine fever

944 **NB:** FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2021.

### Annex 17. Template for curriculum vitae for Reference Laboratory experts

**MEETING OF THE BIOLOGICAL STANDARDS COMMISSION**

**Paris, 5–9 February 2024**

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| | | | |
|---|---|---|---|
| Surname | | Forename(s) | |
| Email address | | Telephone number | |
| Name of the Laboratory | | Disease name | |
| Country of the Laboratory | | Date of submission | |

1. Degrees and qualifications, please provide details and year.

| |
|---|
| |

2. Relevant experience including posts held, with dates and responsibilities (demonstrating experience in laboratory diagnostics)

| |
|---|
| |

3. Information demonstrating international recognition of your expertise: appointments, awards, membership on committees and working groups (relevant to the disease for which you are applying for designation)

|  |
|---|
|  |

4. Publications in peer-reviewed journals and papers in press, related to the disease or pathogen for which you are applying for designation (*Please provide those publications that emphasise your expertise in the specific-disease: **bold** your name in the title of your publications and the pathogen in question*)

| *Number of publications as first author:*<br>*Number of publications as last author:*<br>*Number of publications in other positions:*<br><br>*Please provide the full list of publications in chronological order* |
|---|

**Annex 18. WOAH Procedure for Registration of Diagnostic Kits Validation Studies Abstract (Genelix™ ASFV Real-time PCR Detection kit)**

**MEETING OF THE BIOLOGICAL STANDARDS COMMISSION**

**Paris, 5–9 February 2024**

______________

**Name of the diagnostic kit**: **Genelix™ ASFV Real-time PCR Detection kit**

**Manufacturer: Sanigen Co., Ltd.**

**Procedure /Approval number**:

**Date of Registration**: 052131

**Disease: African Swine Fever (ASF)**

**Pathogen Agent: ASF Virus**

**Type of Assay: Real-time PCR**

## Purpose of Assay

The Genelix™ ASFV Real-time PCR Detection kit is a product that qualitatively detects and confirms the diagnosis of ASFV using a real-time PCR detection system in the whole blood, serum, and tissues of swine suspected of being infected with the ASFV.

### Species and Specimens

The target species is domestic swine, and samples of whole blood, serum, and tissues can be used for testing. Whole blood stored with anticoagulants can be used for the tests. It is recommended that specimens should be tested as soon as possible after collection. However, if immediate use is not achievable, the specimens can be stored for a few days at 4°Cin a fridge or more than seven days at colder than –70℃ in a deep freezer. Specimens should be divided into amounts required for tests and stored at -20±5℃ in a freezer to avoid thawing repeatedly. If the processing or transport is delayed more than 24 hours, it should be kept at –20±5℃. Avoid repeated freeze and thaw.

1. **Information on the kit**

   Please refer to the kit insert available on the WOAH Registry web page or contact the manufacturer at Sanigen Co., Ltd.

   Tel: +82-1833-8010

   Fax: +82-2-573-3134

2. **Summary of validation studies**

## Analytical specificity

***Conclusion***: The interference reaction tests using the positive and the negative samples with five types of interfering substances indicate no interference with results. The cross-reactivity test was evaluated to distinguish between the target and non-target analytes. Exclusivity was confirmed with pathogens related to swine disease or infectious reagents (41 materials, including 16 bacteria, seven swine disease relative viruses, and 18 other viruses). No significant cross-reactivities were found. Nine genotypes of the ASFV p72 gene, the target analyte for the kit, were synthesised and tested for inclusivity. As a result, all types of the gene were detected as positive.

### Analytical sensitivity

***Conclusion:*** The limit of detection (LOD) test of the Genelix™ ASFV Real-time PCR detection kit was performed to measure analytical sensitivity. The significant low positive concentrations were repeated 24 times, and the data were reanalysed using probit analysis in 95 % confidence; as a result, the maximum estimate of 16.9 ($1.7 \times 10^1$) copies/µl was reported as the LOD.

### Repeatability

***Conclusion***: The repeatability was conducted with one person, one lot, for 20 days, with two runs per day, duplication per run, and three different concentrations. As a result of experiments by diluting the ASFV plasmid DNA to the three levels of sample concentration, 100 % of all samples were detected, and negative control showed no amplification in all samples. The coefficient of variation (CV) value was less than 5 % in all cases.

### Diagnostic characteristics

Threshold determination and Diagnostic sensitivity (DSe) and specificity (DSp) estimates:

**Conclusion:**

***Threshold determination:*** The threshold (cut-off) of the Genelix™ ASFV Real-time PCR detection kit is 38.1 Ct. For LOD determined by probit analysis, the cut-off is defined as the average Ct value of the next most concentrated dilution tested to the LOD defined by probit. In the cut-off evaluation, the average Ct value was 38.1 at $2.8 \times 10^1$ copies/µl, which is the closest concentration above the probit value.

**Interpretation of the result**

- The criteria for setting threshold and baseline according to the equipment are as follows.

| Instrument | Threshold | Baseline start | Baseline end |
|---|---|---|---|
| AB 7500 | 0.1 | 3 | 15 |
| AB 7500 Fast | 0.1 | 3 | 15 |
| QuantStudio™ 5 | 0.4 | 3 | 15 |
| Bio-rad CFX96™ | 100 | 3 | 15 |

- If the positive and negative control results match the following criteria listed in the table, interpret the results for the target sample(s). If the results of the control materials do not match the table, repeat the experiment.

| Control type | Ct value |
|---|---|
| Positive Control | Ct ≤ 38.1 |
| Negative Control | Non-Detected |

- Check the Ct value of the sample(s) using the instrument-specific software. The sample data is considered positive at Ct ≤ 38.1 and negative at Ct > 38.1.

**Diagnostic sensitivity (DSe) and specificity (DSp) estimates and 95% confidence intervals.**

- To evaluate the diagnostic sensitivity and specificity, a comparative test was conducted using the reference method (validated and certified by the WOAH), and the results are depicted below.

| Genelix™ ASFV Real-time PCR detection kit | | ASFV/Swine Whole blood & Serum |
|---|---|---|
| Diagnostic sensitivity | N<br>DSe<br>CI | 187<br>99.47 %<br>97.07 to 99.99% |
| Diagnostic specificity | N<br>DSp<br>CI | 553<br>100 %<br>99.33 to 100.0% |

| Genelix™ ASFV Real-time PCR detection kit | | ASFV/Swine Tissue |
|---|---|---|
| Diagnostic sensitivity | N<br>DSe<br>CI | 22<br>100%<br>84.56 to 100.0% |
| Diagnostic specificity | N<br>DSp<br>CI | 450<br>100%<br>99.18 to 100.0% |

## Reproducibility

***Conclusion:*** Three WOAH Reference Laboratories for ASF conducted a comparison study on reproducibility. For the reproducibility of the test, three labs, three days, and two runs per day were compared. All qualitative results were 100% in agreement and met the acceptance criteria with less than CV 5%. The test results are shown in the table below:

| | Coefficients of Variation (%) | | | |
|---|---|---|---|---|
| **Sample No.** | **Sanigen** | **Lab A** | **Lab B** | **Lab C** |
| SNG-01 | 1.09 | 0.46 | 0.90 | 1.36 |
| SNG-02 | 0.68 | 2.81 | 0.43 | 1.19 |
| SNG-03 | 0.40 | 0.40 | 0.40 | 2.42 |
| SNG-04 | 0.68 | 0.92 | 2.56 | 1.66 |
| SNG-05 | 2.20 | 2.86 | 1.86 | 2.28 |
| SNG-06 | Negative | Negative | Negative | Negative |
| SNG-07 | Negative | Negative | Negative | Negative |
| SNG-08 | 0.87 | 2.36 | 1.64 | 0.92 |
| SNG-09 | 0.21 | 4.98 | 2.07 | 0.45 |
| SNG-10 | 0.63 | 1.66 | 1.29 | 0.64 |
| SNG-11 | 0.60 | 0.57 | 0.62 | 1.29 |
| SNG-12 | 0.90 | 1.55 | 0.95 | 0.33 |
| SNG-13 | 0.19 | 2.12 | 0.47 | 0.69 |
| SNG-14 | 0.36 | 0.91 | 0.92 | 1.41 |
| SNG-15 | Negative | Negative | Negative | Negative |
| SNG-16 | 0.60 | 5.18 | 1.07 | 0.78 |
| SNG-17 | 1.04 | 0.42 | 0.43 | 1.00 |
| SNG-18 | 1.03 | 2.07 | 1.02 | 1.08 |
| SNG-19 | 1.02 | 6.13 | 1.54 | 1.71 |
| SNG-20 | Negative | Negative | Negative | Negative |

## Reference


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13. Addressing African swine fever (FAO, 2020)

**Annex 19. WOAH Procedure for Registration of Diagnostic Kits Validation Studies Abstract (Sentinel® ASFV Antibody Rapid Test)**

**MEETING OF THE BIOLOGICAL STANDARDS COMMISSION**

**Paris, 5–9 February 2024**

______________

**Name of the diagnostic kit**: Sentinel® ASFV Antibody Rapid Test

**Manufacturer**: Excelsior Bio-System Incorporation

**Procedure /Approval number**: 062233

**Date of Registration**:

**Disease:** African swine fever

**Pathogen Agent:** African swine fever virus

**Type of Assay:** Immuno-chromatographic lateral flow assay (Rapid test)

**Purpose of Assay:** Detection of antibody associated with current infection or an immune response to previous exposure in an individual animal, group of animals or defined population. For use in conjunction with other tests or diagnostic procedures, as an aid in diagnosis or other clinical or epidemiological assessments.

**Species and Specimens:** Porcine serum

## 1. Information on the kit

Please refer to the kit insert available on the WOAH Registry web page or contact manufacturer at:

Website link: ebs.com.tw/en/products/asfvrt

Email address: sales@ebs.com.tw

## 2. Summary of validation studies

### Analytical specificity

***Conclusion***:

**a)** Sentinel® ASFV Antibody rapid test can be used for serum sample from different genotypes (I, II, IX, X) of African swine fever virus infection.

**b)** Sentinel® ASFV Antibody Rapid Test can provide a high-specificity result (93/95 = 97.89%; 95% CI = 92.6% to 99.74%) with a very low cross-reactivity for 95 individual samples from 19 typical pig pathogens (non ASFV) of the domestic pigs.

**c)** Potential interfering factors, such as anticoagulants, haemolysis (haemoglobin) and lipaemia (intralipid), did not affect the test results.

## Analytical sensitivity

***Conclusion:***

There was more than 80% agreement between the EURL-IPT test and Sentinel test when the sera had antibody titres higher than 1:5120.

## Repeatability

***Conclusion***:

For the intra-assay, an operator evaluated 4 reference sera (strong, medium, weak, and negative) in quadruplicate tests. Inter-assay agreement was evaluated using the same 4 reference sera in 20 runs by three operators on separate days with different batches of kits. All intra-assay and inter-assay runs of the four reference sera produced identical results. The Sentinel® ASFV Antibody Rapid Test demonstrated 100% repeatability. According to the European Reference Laboratory (EURL) intra-assay and inter-assay reports, 10 reference sera were tested in one round/day for 2 days, and each round was tested in duplicate. The Sentinel® ASFV Antibody Rapid Test had 100% repeatability.

## Diagnostic characteristics:

**Threshold determination:**

Sentinel® ASFV Antibody Rapid Test is a qualitative test. The test sample is positive when two lines (C line and T line both) appear and negative when only the C line appears. The threshold (cut-off) of antibody titre is > 1:640 (>50% agreement with EURL-IPT test).

**Diagnostic sensitivity (DSe) and specificity (DSp) estimates:**

788 serum samples have been tested. The results obtained from EURL and Excelsior Bio-System evaluation report.

| | EURL-IPT | | ASFV free |
|---|---|---|---|
| | Positive | Negative | Negative |
| Category 1: EURL-ASF-Ref1 | 8 | 2 | – |
| Category 2: Reference experimental serum | 122 | 23 | – |
| Category 3: Experimental samples from pigs infected with genotype II ASFV | 148 | 96 | – |
| Negative serum samples from National Pingtung University of Science and Technology (NPUST), Taiwan | – | – | 389 |
| Total | 278 | 121 | 389 |

| Sentinel® ASFV Antibody Rapid Test | | Specimens |
|---|---|---|
| Diagnostic Sensitivity (DSe) | **81.65%**<br>(95% CI = 76.60% to 86.02%) | EURL-IPT Positive: 278 |
| Diagnostic Specificity (DSp) | **96.27%**<br>(95% CI = 94.24% to 97.74%) | EURL-IPT Negative:121<br>NPUST ASFV Free: 389 |

## Reproducibility

***Conclusion***:

The reproducibility study was performed by the Pirbright Institute and evaluated in three laboratories. 22 positive and 20 negative samples, as determined by ELISA (the reference standard), were tested. The results indicate the Sentinel® ASFV Ab Rapid Test can produce results with a reasonable degree of reproducibility when used to test replicate samples in different laboratories. The kappa values of interlaboratory comparison are following.

| Interlaboratory | Kappa Value | Result |
|---|---|---|
| Lab 1 and Lab 2 | **0.781**<br>(95%CI = 0.582 to 0.981) | substantial agreement |
| Lab 1 and Lab 3 | **0.850**<br>(95%CI = 0.695 to 1.000) | very high agreement |
| Lab 2 and Lab 3 | **0.791**<br>(95%CI = 0.603 to 0.979) | substantial agreement |

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