

Report of the Meeting of the WOAH Biological Standards Commission

Original: English (EN)

8 to 12 September 2025

Paris

Introduction and Member contribution

This report presents the work of the WOAHA Biological Standards Commission (hereinafter called 'the Commission'), which met in Paris, France from 8 to 12 September 2025.

During the meeting, 17 chapters from the WOAHA *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (*Terrestrial Manual*) were approved for circulation for first-round Member comment, nine Reference Centre applications and nine nominations for replacement experts were also evaluated.

Please note that the texts for comment are presented in [Annexes 4 to 20](#).

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

Annexes

Texts in [Annexes 4 to 20](#) are presented for first-round comment.

How to submit comments

The Biological Standards Commission strongly encourages WOAHA Members and International Organisations with a WOAHA Cooperation Agreement to participate in the development of WOAHA 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 that standards are science-based and take into consideration the different contexts among Members and stakeholders and can be implemented. To ensure that comments are considered, they should be submitted by the requested deadline and in the format described in the [Guide for WOAHA Members and International Organisations on submitting comments during the process for the elaboration of WOAHA International Standards](#) (Guide) and the [Standard Operating Procedure for WOAHA Members and International Organisations to submit comments during the process for the elaboration of WOAHA International Standards](#) (SOP) available on the Delegate's website and the WOAHA public website.

Comments that are not correctly formatted as described in the [Guide](#) and [SOP](#) will 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

As highlighted by the President of the Commission at the General Session, the purpose of the first round of commenting is for Members to submit their experts' in-depth reviews that will improve the scientific accuracy and relevance of the chapters. Following the February meeting, the chapters are sent for second-round comment as the versions that will be presented for adoption. Second-round comments should be only essential amendments that are crucial to the update.

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 WOAHA website at [Ad hoc Groups - WOAHA - World Organisation for Animal Health](#).

Deadline to comment

Comments on relevant texts in this report must reach the Headquarters by [27 December 2025](#) 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 Commission noted the dates for its next meeting: [2 to 6 February 2026](#)



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1. Welcome

1.1. Director General

Dr Emmanuelle Soubeyran, WOA Director General, and Dr Baba Soumaré, WOA Deputy Director General for Global Framework and Capacity Building, welcomed the members of the Commission.

Dr Soubeyran opened the meeting by introducing Dr Soumaré in his new role, underlining his in-depth technical expertise, extensive leadership experience and his inspiring vision. She noted his conviction that establishing capacity is fundamentally about building institutional trust, enabling scientific progress and ultimately improving the lives of both animals and humans.

In his first remarks as Deputy Director General, Dr Soumaré expressed his gratitude for the opportunity to work with the Commission. He acknowledged the central role of the Commission in the standard-setting process and in ensuring a solid scientific foundation for the work of WOA. He emphasised the importance of continual transformation and improvement to better serve Members, pointing out that the Specialist Commissions constitute the backbone of the Organisation's science-based decisions. He highlighted the fact that when epidemiological events require rapid action, it is this Commission and the network of experts that provide the guidance and scientific validation needed. He concluded by stressing that WOA's strong commitment to supporting the work of the Commission.

The Director General presented WOA's new organisational structure, noting the increased delegation of responsibilities and the enhanced transversality among departments at the Headquarters. She recalled the Organisation's 8th Strategic Plan, which sets out three overarching objectives: to develop adaptable and science-based standards, to empower Members to strengthen resilient animal health systems, and to place animal health at the centre of global priorities. She further explained that the plan emphasises governance and sustainability, placing people at the heart of the Organisation's mission through expertise, capacity building and data-driven approaches. She underscored the importance of accountability, highlighting that WOA is reinforcing its internal monitoring and evaluation systems to ensure effective oversight of its activities.

The Director General also addressed the work of the Governance Review Committee, which is currently examining proposals concerning governance, finance and technical matters. She acknowledged that some of these proposals may appear innovative, but she insisted on the importance of safeguarding what already functions effectively, while at the same time carefully considering the reasoning behind proposed changes.

Dr Soubeyran warmly congratulated the Commission for its significant achievements, in particular the adoption of 28 chapters of the *Terrestrial Manual* during the 92nd General Session. She recognised the collective effort and expertise that made this possible, noting especially the successful revision of demanding chapters such as those on African swine fever (ASF) and avian influenza (AI). She reminded the members that the adopted versions of these chapters were available through the new [Standards Online Navigation Tool](#).

She also underscored the importance of the WOA Reference Centres, highlighting the adoption of three new Reference Laboratories and five new Collaborating Centres, as well as the successful Reference Centre kiosk organised during the General Session. She reiterated the vital role of visibility and recognition of Reference Centres, describing them as the cornerstone of WOA's scientific expertise. Dr Soubeyran encouraged the Commission to continue its dedicated efforts in this regard.

Finally, Dr Soubeyran informed the Commission that next year's General Session will take place in a new venue, in La Défense – Paris, which will allow for the inclusion of a greater number of side events. She also confirmed that the General Session is scheduled to begin on **18 May 2026**.

The members of the Commission expressed their appreciation for the updates shared by the Directorate and conveyed their best wishes to Dr Soumaré for success in his mandate as Deputy Director General.

1.2. Joint meeting with all Specialist Commissions – 30 Sept 9h00 CET or 6 Oct 16h00 CET (virtual)

1.2.1. WOA 8th Strategic Plan

The Commission was informed that the WOA 8th Strategic Plan (2027–2031) is currently under development and will be presented for adoption at the May 2026 General Session. The process follows a phased approach. The first phase involved internal consultations and a review of the 7th Strategic Plan which resulted in three strategic orientations: development of global standards adaptable and applicable to all, empowering Members towards robust animal health and welfare systems and veterinary public health, and positioning animal health as central to tackling global challenges. Four enablers were identified to support the strategic orientations,

including governance and partnerships for sustainability, leadership in animal health data, people at the centre of WOAAH and performance and accountability. The second phase saw the expansion of consultations to WOAAH Members and staff.

The third and current phase involves continued drafting and review of the 8th Strategic Plan. A draft will be presented to the WOAAH Council in October 2025, after which it will be shared with stakeholders, including the Specialist Commissions, for feedback. A revised draft will then be presented to the Council in early 2026 ahead of the General Session in May 2026.

1.2.2. Recommendations of the Governance Review Committee

The Commission was updated of the work of the WOAAH Governance Review Committee in developing recommendations to the Assembly on updates to WOAAH's legal framework, institutional, regional, technical and financial governance in accordance with Assembly [Resolution No. 12 \(2024\)](#) and [Resolution No. 5 \(2025\)](#). Relevant to the work of WOAAH Specialist Commissions, the work included a review of the appointment processes of experts to WOAAH's Specialist Commissions and other technical bodies, the development of a Technical Procedural Manual on WOAAH's technical decision-making processes and distinguishing binding and advisory standards, with final recommendations expected to be presented at the WOAAH 93rd General Session. Specialist Commission members were encouraged to engage with their Delegate on the Governance Review Committee's work.

1.2.3. Animal Health Forum

The Commission was updated on the Animal Health Forum held during the 92nd WOAAH General Session, which focused on veterinary vaccines and vaccination, and addressed barriers to vaccine availability, access, demand and adoption. Discussions covered trade implications, regulatory challenges, public perception, and the role of vaccines in reducing antimicrobial resistance and supporting One Health. The Forum informed the adoption of [Resolution No. 29](#), which outlined 11 recommendations for WOAAH and its Members to enhance vaccine availability, quality, and strategic use. The technical report of the session may be found [here](#). The Commission was invited to review the recommendations and provide WOAAH Headquarters with feedback on the implementation of the Resolution.

1.2.4. Global Conference on Biological Threat Reduction

The Commission was informed that WOAAH's Global Conference on Biological Threat Reduction (Geneva, Switzerland, 28–30 October 2025) will bring together 400 participants from sectors including animal health, public health, and law enforcement to address the evolving landscape of biological threats. Coinciding with key anniversaries of the Biological Weapons Convention and Geneva Protocol, the Conference aims to reflect on historical and current biological threats and anticipate future risks, strengthen international cooperation and secure concrete commitments, foster multi-sectoral collaboration for global health security and promote innovative strategies linking animal health with other sectors.

The Conference will feature plenaries, panels, and side events, with expected outcomes including stronger partnerships and improved biothreat awareness.

1.3. Transparency of commenting – phased approach

As originally communicated in the Commission's September 2023 report, the Director General agreed to progressively implement a process to improve the transparency of the WOAAH process for the elaboration of Standards for better documentation and traceability.

The first step in this process was the publication on the Delegates' website of comments considered by the Commission at its February 2024 meeting.

The second step in this process was the publication on the Delegates' and public WOAAH websites of comments considered by the Commission at its September 2024 meeting. The Commission also included its responses to comments in Annex 4 for information only. As a result, the Commission's report was more concise and focused on providing a short background summary for each agenda item as well as key points discussed and agreed by the Commission.

The third and final step in this process will be to publish the name of the Member or partner organisation who submitted the comment considered by the Commission at its February 2026 meeting, linked with the comment provided in Annex 4.

These new processes are aimed at improving transparency of the standard-setting process and creating a more user-friendly report. Together with the guidance documents (see 'How to submit comments' in this report) for submitting comments that are available for Members and partners, we hope that more Members can actively engage in the process for the elaboration of WOAHS Standards.

2. Adoption of the agenda

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

3. Liaison with other Specialist Commissions

3.1. Terrestrial Animal Health Standards Commission

3.1.1. Glossary term: laboratory

At its February 2023 meeting, the Terrestrial Animal Health Standards Commission (TAHSC) agreed to draft a new *Terrestrial Code* Article (1.6.4.) clarifying that the presence of pathogenic agents in an approved laboratory, maintained with appropriate levels of containment and biosecurity as outlined in the *Terrestrial Manual*, does not affect the animal health status of a country or zone.

In February 2024, the TAHSC considered feedback from the Scientific Commission for Animal Diseases (Scientific Commission) and concurred that the existing Glossary definition of "laboratory" was limited to diagnostic purposes. It therefore agreed to broaden the scope to include other types of approved animal facilities, such as those engaged in research, vaccine development and manufacturing, or biological production requiring the handling of pathogenic agents.

In February 2025, the Commission reviewed ongoing discussions on this matter and recognised the need for a more comprehensive definition that encompasses facilities beyond diagnostic functions, while ensuring consistency with biosafety requirements and existing standards, and proposed to provide a revised definition for consideration at its bureaux meeting in September 2025.

The draft definition proposed by the Commission emphasises that laboratories may be involved in diagnosis, research, vaccine and diagnostic material production, or other biological activities, with due requirements for infrastructure, staffing and technical competence. The Commission further noted the importance of explicitly recognising the oversight role of a specialist in research, veterinary diagnostics or vaccines, as this provides clear accountability and aligns with the WOAHS expert framework.

The draft definition was discussed in the Bureaux meeting, the TAHSC thanked the Commission for the support on the proposed definition.

3.1.2. Advice to the TAHSC regarding a Member comment on draft Code chapter on Crimean–Congo haemorrhagic fever

See agenda item 5.7. of the report of the meeting of the meeting of the TAHSC, 9–18 September 2025.

3.1.3. Bureau meeting

See agenda item 3.2. of the report of the meeting of the meeting of the TAHSC, 9–18 September 2025.

3.2. Scientific Commission for Animal Diseases

3.2.1. Case definitions: infection with *Chlamydia abortus*, anthrax and Aujeszky's disease

The Commission discussed the case definitions for infection with *Chlamydia abortus*, anthrax and Aujeszky's disease and gave its recommendations to the Scientific Commission.

Regarding the case definition for enzootic abortion of ewes, the Commission was presented with the draft case definition for *Chlamydia abortus*. The Commission reviewed the diagnostic criteria, in particular for the detection of antigen, nucleic acid and antibodies and agreed with the experts to exclude epidemiological links to suspected cases, as clinical signs for *C. abortus* are not pathognomonic and these tests should be accompanied with supporting evidence such as clinical signs and pathological lesions of the animal, or

otherwise with an epidemiological link to a confirmed case. The Commission noted that this is a change that would require harmonisation across the disease-specific chapters and proposed to discuss this further with the TAHSC.

Regarding the case definition for anthrax, the Commission was presented with the draft case definition for *Bacillus anthracis*. The Commission agreed with the experts' opinion that it would be recommended to refer to the pathogenic agent as *B. anthracis* as referenced in the *Terrestrial Manual*, also with the view that bovines, caprines, antilopinae, camelids, equids and suids are relevant animal hosts in the occurrence of a case. Finally, the Commission agreed with the experts to exclude seroconversion from the diagnostic criteria. The Commission also noted that in some cases, human cases may be the first indication of anthrax in the geographical area. Taking reference from Chapter 8.15. 'Infection with Rift Valley fever virus', the Commission proposed to include reference to human cases as supporting evidence to classify an antigen or nucleic acid-positive animal as a case.

Regarding the case definition for Aujeszky's disease, the Commission was presented with the draft case definition for infection with suid alphaherpesvirus 1. The Commission agreed with the experts' opinion that it would be recommended to refer to the pathogenic agent as suid alphaherpesvirus 1 as referenced in the *Terrestrial Manual*. The Commission also agreed with the experts with the diagnostic criteria proposed by the experts (isolation, detection of antigen, nucleic acid, seroconversion and antibody) to confirm a case of infection with suid alphaherpesvirus 1.

For details of the case definition, refer to agenda item 8.2.1, 8.2.2. and 8.2.3. of the report of the meeting of the Scientific Commission, 8–12 September 2025.

3.2.2. Guidelines for African swine fever vaccines: field evaluation and post-vaccination monitoring

Following the adoption of Section C of *Terrestrial Manual* chapter 3.9.1 on ASF, the Commission was informed that in response to Members' requests, WOAHA had launched a consultancy to develop a set of guidelines on the field evaluation of ASF vaccines and how to conduct post-vaccination monitoring in accordance with WOAHA international standards. The primary target audience for the guidelines would include Competent Authorities involved in the regulatory approval of veterinary vaccines and Veterinary Services responsible for the implementation of ASF control strategies.

The first draft of the guidelines developed by the consultants was peer-reviewed by an *ad hoc* Group that met from 22 to 24 July 2025. The Group's recommendations were shared with the Commission and the Scientific Commission for Animal Diseases. The report of the *ad hoc* Group is available [here](#). Both Commissions were invited to provide their feedback on the second draft of the guidelines.

The Commission noted that the Group's recommendations are relevant to other diseases and the growing need for harmonised standards to guide the design, implementation, and interpretation of vaccine field trials. It further noted that a robust post-vaccination monitoring framework is essential to assess vaccine effectiveness, safety, and impact once deployed in the field. The Commission supported the Group's proposal to draft a new *Terrestrial Manual* chapter on post-vaccination monitoring and will consider the best approach to implementing this proposal at the February 2026 meeting.

The Commission designated a member to review all the chapters in Section 2.3; 'Veterinary vaccines' of the *Terrestrial Manual* with the aim of exploring the best way to address the multiple proposals for inclusion of information on veterinary vaccines. The Commission will decide the way forward at the February 2026 meeting following the report of this analysis.

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 John Pasick, Consultant Editor of the WOAHA *Terrestrial Manual*.

5.1. Review of draft chapters and their endorsement for circulation for first-round Member comment

The Commission reviewed 18 draft chapters and approved 17 for circulation, some subject to clarification of certain points by the experts, for first-round Member comment and eventual proposal for adoption by the World Assembly of WOAHA Delegates in May 2026.

Chapter 1.1.2. 'Collection, submission and storage of diagnostic specimens': the Commission reviewed chapter 1.1.2., which had been updated by a WOAHP Working Group on Wildlife to include information relevant to wildlife. The experts updated it to include information on sampling in wildlife species.

The revised Chapter 1.1.2. 'Collection, submission and storage of diagnostic specimens' is presented as [Annex 4](#) for first-round Member comment.

The main amendments include:

Section/paragraph	Change
A. 'Collection of samples'	Changed "sample" to "specimen" in the Section title and throughout the chapter, where appropriate, to reflect the adopted definitions of these terms as given in glossary of the <i>Terrestrial Manual</i>
	Added information on considerations to be taken into account when sampling and transporting specimens from wild species
	Added new subsections on sampling urine and arthropod insects
C. 'Information to be sent with specimens'	Reinforced the recommendations on shipping of specimens from the field to the laboratory
D. 'Receipt, storage and archives of laboratory submissions'	Added a new subsection on specimen quality
References	Updated the list of references

Chapter 1.1.3. 'Transport of biological materials': the Commission reviewed chapter 1.1.3., which had been updated by a WOAHP Member following a request to include guidance on vaccine cold chain management in the *Terrestrial Manual* (see item 3.1.1. 'Guidelines on vaccine cold chains' of the [report of the February 2025 BSC meeting](#)).

The revised Chapter 1.1.3. 'Transport of biological materials' is presented as [Annex 5](#) for first-round Member comment.

The main amendments include:

Section/paragraph	Change
Throughout the chapter	Reviewed and amended, where necessary, the terms "sample" and "specimen" throughout the chapter to reflect the adopted definitions of these terms as given in glossary of the <i>Terrestrial Manual</i>
C.7. 'Cold chain transportation'	Added a new subsection on cold chain requirements for vaccines in response to a request from a WOAHP Member

Chapter 3.1.8. 'Foot and mouth disease (infection with foot and mouth disease virus)' **appendices only**: the Commission reviewed the newly draft appendices to chapter 3.1.8., which had been drafted by the WOAHP Reference Centre experts. The appendices include the justification for the scores given to each test method listed in Table 1 of the chapter.

The newly drafted appendices to Chapter 3.1.8. 'Foot and mouth disease (infection with foot and mouth disease virus)' are presented as [Annex 6](#) for first-round Member comment.

The main amendments include:

Section/paragraph	Change
Appendices 1 to 6	Added six appendices justifying the choice of assays and their ratings given in Table 1
References	Updated the list of references accordingly

Chapter 3.1.11. 'Leishmaniosis': the Commission reviewed chapter 3.1.11., which had been updated by the WOA Reference Laboratory expert.

The revised Chapter 3.1.11. 'Leishmaniosis' is presented as [Annex 7](#) for first-round Member comment.

The main amendments include:

Section/paragraph	Change
A. 'Introduction'	Thoroughly updated this Section to conform with the chapter template, including adding a subsection on nature and classification of the pathogen and deleting the subsection on the role of the vector.
B. 'Diagnostic techniques'	Added a new Section 'Diagnostic objectives for feline leishmaniosis' along with Table 2 'Test methods available for diagnosis of feline leishmaniosis and their purpose' and an accompanying appendix
B.1.4. 'Molecular methods – detection of nucleic acid'	Thoroughly updated the information on real-time and conventional PCRs and reformatted using the tables of the primer/probe sequences and cycling parameters to follow the chapter template
C. 'Requirements for vaccines and diagnostic biologicals'	Thoroughly updated this Section by adding background information and expanding the subsection 'Outline of production and minimum requirements for vaccines' with more details on available vaccines. Deleted the subsection 'Diagnostic biologicals (antigens for skin tests)' as no recommendations can be made by WOA
Appendices 1 to 6	Added six appendices justifying the choice of assays and their ratings given in Table 1
References	Thoroughly updated the reference list

Chapter 3.1.17. 'Paratuberculosis': the Commission reviewed chapter 3.1.17., which had been updated by the WOA Reference Laboratory experts.

The revised Chapter 3.1.16. 'Paratuberculosis' is presented as [Annex 8](#) for first-round Member comment.

The main amendments include:

Section/paragraph	Change
Summary	Rearranged the text for clarity and to follow the chapter template
A. 'Introduction'	Reformatted the text and added subheadings to follow the chapter template
Table 1 'Test methods available for diagnosis of paratuberculosis and their purpose'	Revised the scores of many of the tests in the Table for different purposes and added a footnote clarifying that for the purpose 'Immune status in individual animals or populations post-vaccination' test results at the individual level are of limited value in distinguishing between infection and

Section/paragraph	Change
	vaccination whereas at the herd level, aggregating results increases reliability and, under specific circumstances, allows more confident inferences to be made about whether a herd has been vaccinated
B.2.1. 'Enzyme-linked immunosorbent assay'	Revised the order of the paragraphs, deleted some out-of-date text and added new information on variation in the sensitivity and specificity of milk and serum ELISA at the herd level: cross reactions after the intradermal comparative cervical tuberculin test has been reported, the impact of which should be evaluated for each control programme.
B.2.2. 'Complement fixation test'	Deleted the CFT because it is no longer used as it is very laborious, works well only on clinically suspect animals, and does not have sufficient specificity to enable its use in the general population for control purposes
References	Updated the list of references
Appendices 1 to 6	Added six appendices justifying the choice of assays and their ratings given in Table 1

Chapter 3.3.3. 'Avian infectious laryngotracheitis': the Commission reviewed chapter 3.3.3., which had been updated by disease experts.

The revised Chapter 3.3.3. 'Avian infectious laryngotracheitis' is presented as [Annex 9](#) for first-round Member comment.

The main amendments include:

Section/paragraph	Change
B.1.2. 'Molecular methods – detection of nucleic acid'	Updated the Section and reformatted using the tables of the primer/probe sequences and cycling parameters for the real-time and conventional PCRs to follow the chapter template
References	Updated the list of references
Appendices 1 to 6	Added six appendices justifying the choice of assays and their ratings given in Table 1

Chapter 3.3.5. Avian mycoplasmosis (*Mycoplasma gallisepticum*, *M. synoviae*): the Commission reviewed chapter 3.3.5., which had been updated by the WOA Reference Laboratory experts.

The revised Chapter 3.3.5. 'Avian mycoplasmosis (*Mycoplasma gallisepticum*, *M. synoviae*)' is presented as [Annex 10](#) for first-round Member comment.

The main amendments include:

Section/paragraph	Change
A.1. 'Description and impact of the disease'	Added information on the role of environmental factors in increasing the number of lesions reported during MG infection. Other upper respiratory pathogens (such as Newcastle disease virus), as well as live virus vaccinations also contribute to the exacerbation of MG infection clinical signs
B.1.3. 'Molecular methods – detection of nucleic acids'	Updated and reformatted the Section using the tables of the primer/probe sequences and cycling parameters for the real-time and conventional PCRs to follow the chapter template

Section/paragraph	Change
B.2. 'Serological tests'	Added text to the introduction to this Section explaining why any serological screening conducted using blood collected less than 10 days post-hatching should be considered a screening of the layer hens rather than the chicks
References	Updated the list of references
Appendices 1 to 6	Added six appendices justifying the choice of assays and their ratings given in Table 1

Chapter 3.3.7. 'Fowl typhoid and Pullorum disease': the Commission reviewed chapter 3.3.7., which had been updated by the WOA Reference Laboratory experts on salmonellosis.

The revised Chapter 3.3.7. 'Fowl typhoid and Pullorum disease' is presented as [Annex 11](#) for first-round Member comment.

The main amendments include:

Section/paragraph	Change
A. 'Introduction'	Added a statement that the pathogens are increasingly associated with antimicrobial resistance, including multidrug-resistant strains resistant to cephadrine, trimethoprim-sulfamethoxazole, and ampicillin
A.1. 'Zoonotic risk and biosafety requirements'	Updated the information on human illness due to <i>S. Pullorum</i> . Added a statement a statement that systemic human infection with <i>S. Gallinarum</i> has rarely been documented and that no evidence is available of interpersonal transmission of these biovars
B.1.1.2. 'Liquid enrichment and selective media'	Added a statement that selenite cysteine medium should be used in addition to Rappaport–Vassiliadis soya (RVS) broth and Muller-Kauffmann Tetrathionate-Novobiocin Broth (MkTTn) broth for the detection of <i>S. Gallinarum</i> and <i>Pullorum</i> biovars; appropriate protective precautions should be taken when preparing and handling sodium hydrogen selenite
B.1.5. 'Molecular epidemiology'	Highlighted that whole-genome sequencing is the gold standard for typing isolates and for evaluating their genetic correlation
References	Updated the list of references
Appendices 1 to 6	Added six appendices justifying the choice of assays and their ratings given in Table 1

Chapter 3.4.4. 'Bovine genital campylobacteriosis': the Commission reviewed chapter 3.4.4., which had been updated by WOA Reference Laboratory experts.

The revised Chapter 3.4.4. 'Bovine genital campylobacteriosis' is presented as [Annex 12](#) for first-round Member comment.

The main amendments include:

Section/paragraph	Change
Table 1. Test methods available for diagnosis of bovine genital campylobacteriosis and their purpose	Added a footnote clarifying that culture is the gold standard, but that there are no data on specificity and sensitivity at the herd level. The characteristics of the additional tests described in the chapter are compared with culture at the sample level, not at the herd level

Section/paragraph	Change
B.1.7. 'Biochemical identification of <i>Campylobacter fetus</i> subspecies'	Updated this Section and Table 2. 'Differential characteristics of <i>Campylobacter</i> species potentially isolated from the bovine genital tract and aborted fetuses' to remove <i>C. jejuni</i> and growth at 25°C and 42°C because there are no diagnostic methods that subspeciate <i>C. jejuni</i> , and because growth at these temperatures does not conclusively differentiate <i>Campylobacter</i> spp.
B.1.9. 'Molecular methods – detection of nucleic acids'	Updated and reformatted the Section using the tables of the primer/probe sequences and cycling parameters for the real-time and conventional PCRs to follow the chapter template
References	Updated the list of references

Chapter 3.4.10. 'Haemorrhagic septicaemia (*Pasteurella multocida* serotypes 6:b and 6:e)': the Commission reviewed chapter 3.4.10., which had been updated by a subject matter expert.

The revised Chapter 3.4.10. 'Haemorrhagic septicaemia (*Pasteurella multocida* serotypes 6:b and 6:e)' is presented as [Annex 13](#) for first-round Member comment.

The main amendments include:

Section/paragraph	Change
A. 'Introduction'	Added a statement that there can be spatial and temporal variation in the occurrence of HS outbreaks within an endemic country, which indicates the need for targeted and tailored prevention and control strategies within endemic settings
B.1.3. 'Molecular methods – detection of nucleic acids'	Updated and reformatted the Section using the tables of the primer/probe sequences and cycling parameters for the real-time and conventional PCRs and loop-mediated isothermal amplification (LAMP) assays to follow the chapter template
B.1.3.4. 'Genotypic differentiation of isolates'	Expanded this Section to include information on virulence-associated genes and virulence genotyping, and on multilocus sequence typing (MLST) schemes that have been developed for the characterisation of <i>P. multocida</i> strains
B.1.3.5. 'Whole genome sequence-based typing'	Added this new Section as it is current and relevant
B.1.4. 'Biomarker-based method'	Added this new Section as it is current and relevant
B.2. 'Serological test'	Added information on the ELISA, which has been employed to detect antibodies against HS in a limited study. The ELISA was also added to Table 1. 'Test methods available and their purpose'
References	Updated the list of references
Appendices 1 to 6	Added six appendices justifying the choice of assays and their ratings given in Table 1

Chapter 3.4.11. 'Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis': the Commission reviewed chapter 3.4.11., which had been updated by the WOA Reference Laboratory experts.

The revised Chapter 3.4.11. 'Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis' is presented as [Annex 14](#) for first-round Member comment.

The main amendments include:

Section/paragraph	Change
A. 'Introduction'	Expanded information on stability of the virus and inactivation methods
B.1.3. 'Molecular methods – detection of nucleic acids'	Updated and reformatted the Section using the tables of the primer/probe sequences and cycling parameters for the real-time and conventional PCRs to follow the chapter template
B.2. 'Serological tests'	Added a statement that the antibody contents of meat juice samples taken from the carcasses of adult fattening bulls after slaughter are 10–30% reduced compared with blood samples, and thus a BoAHV-1 status definition by this method is not possible but an estimation of IBR prevalence in beef cattle herds as well as survey of negative herds is enabled
B.3. 'Emerging rapid diagnostics'	Added a new Section on rapid tests.
References	Updated the list of references
Appendices 1 to 6	Added five appendices justifying the choice of assays and their ratings given in Table 1

Chapter 3.5.1. 'Camelpox': the Commission reviewed chapter 3.5.1., which had been updated by the WOA Reference Laboratory expert.

The revised Chapter 3.5.1. 'Camelpox' is presented as [Annex 15](#) for first-round Member comment.

The main amendments include:

Section/paragraph	Change
Summary	Replaced the terms "Old World" and "New World" [camelids] by the genus names as more suitable for a WOA Standard
B.1.5. 'Molecular methods – detection of nucleic acids'	Updated and reformatted the Section using the tables of the primer/probe sequences and cycling parameters for the real-time and conventional PCRs to follow the chapter template
Appendices 1 to 6	Added five appendices justifying the choice of assays and their ratings given in Table 1

Chapter 3.6.4. 'Equine encephalomyelitis (Eastern, Western and Venezuelan)': the Commission reviewed chapter 3.6.4., which had been updated by a subject matter expert.

The revised Chapter 3.6.4. 'Equine encephalomyelitis (Eastern, Western and Venezuelan)' is presented as [Annex 16](#) for first-round Member comment.

The main amendments include:

Extensively updated chapter
The chapter was extensively updated, with most of the existing text being updated or rearranged for clarity, to provide more up-to-date information on the three viruses individually and also focusing on areas where they differ. All Sections were thoroughly updated, a new Table 3. 'Test methods available for the diagnosis of VEEV and their purpose' was added. The reference Section has also been thoroughly updated and five appendices justifying the choice of assays and their ratings given in Tables 2 and 3 were added.

Chapter 3.6.6. 'Equine influenza (infection with equine influenza virus)': the Commission reviewed chapter 3.6.6., which had been updated by the WOA Reference Laboratory experts.

The revised Chapter 3.6.6. 'Equine influenza (infection with equine influenza virus)' is presented as [Annex 17](#) for first-round Member comment.

The main amendments include:

Section/paragraph	Change
A. 'Introduction'	Added a statement that vaccines containing recombinant canarypox viruses expressing the haemagglutinin (HA) gene from equine influenza virus are widely used, and a nucleoprotein-based ELISA can differentiate infected animals from vaccinates (DIVA)
Table 1 'Test methods available for diagnosis of equine influenza and their purpose'	Revised the scores of many of the tests in the Table for different purposes
B.1. 'Identification of the agent'	Updated some of the tests in this Section, namely haemagglutinin characterisation, neuraminidase characterisation, real-time reverse transcriptase (RT) PCR, and molecular characterisation, and used the tables of the primer/probe sequences and cycling parameters to follow the chapter template; added a new subsection on point-of-care tests for agent identification and characterisation
B.2. 'Serology tests'	Updated some of the tests in this Section, namely the haemagglutination inhibition test, single radial haemolysis, and the competitive/blocking ELISA
C. 'Requirements for vaccines'	Deleted subsection 2.2.4 'Single radial diffusion test' because the reference reagents have not been available for over a decade and the test is not known to be currently used by vaccine companies
C.4. 'Post-vaccination monitoring'	Added a new Section on post-vaccination monitoring
References	Updated the list of references
Appendices 1 to 6	Added six appendices justifying the choice of assays and their ratings given in Table 1

Chapter 3.6.10. 'Glanders and melioidosis': the Commission reviewed chapter 3.6.10., which had been updated by the WOA Reference Laboratory experts.

The revised Chapter 3.6.10. 'Glanders and melioidosis' is presented as [Annex 18](#) for first-round Member comment.

The main amendments include:

Section/paragraph	Change
Summary	Thoroughly updated the Summary especially the description and importance of the disease. In the subsection on 'Serological tests' added a statement that CFT and the newly validated recombinant double-antigen ELISA are recommended for individual animal freedom from infection prior to movement
A. 'Introduction'	Thoroughly updated this Section including adding information on glanders incubation periods, expanding the description of melioidosis, and adding information on survival of the pathogenic agent outside the host

Section/paragraph	Change
Table 1. 'Test methods available for the diagnosis of glanders and their purpose'	Revised the scores given to some tests in Table 1, in particular the ELISA, adding a footnote that only validated ELISAs should be used, especially for mules and donkeys, which are not always included in validation studies.
B.2. 'Detection of the agents'	Updated some of the tests in this Section, namely adding <i>Burkholderia pseudomallei</i> in the subsection on morphology of the pathogenic agent, adding a novel section medium to the subsection on cultural characteristics, updating and reformatting the subsection on identification of <i>Burkholderia mallei</i> and <i>B. pseudomallei</i> by PCR, including using the tables of the primer/probe sequences and cycling parameters to follow the chapter template; added a new subsection on molecular typing methods and whole genome sequencing
B.3. 'Serology tests'	Thoroughly updated the tests in the Section, namely the CFT, the ELISA and the immunoblot assays.
References	Updated the list of references
Appendices 1 to 5	Added five appendices justifying the choice of assays and their ratings given in Table 1 and added a link to the interlaboratory comparison test report comparing the CFT and the newly validated recombinant double-antigen ELISA

Chapter 3.8.3. 'Contagious agalactia': the Commission reviewed chapter 3.8.3., which had been updated by the WOA Reference Laboratory experts.

The revised Chapter 3.8.3. 'Contagious agalactia' is presented as [Annex 19](#) for first-round Member comment.

The main amendments include:

Section/paragraph	Change
Summary	Updated the description and importance of the disease to emphasise that <i>Mycoplasma agalactiae</i> (<i>Ma</i>) is the historical and sole cause of the classical form of the disease in domestic sheep and goats
A. Introduction	Thoroughly update this Section mentioning that the disease is under-reported, describing clinical signs, and explaining that three other mycoplasmas belonging to a phylogenetically distinct group have been added to the aetiology of contagious agalactia because they can cause similar clinical signs, although almost exclusively in goats
Table 1. 'Test methods available for the diagnosis of contagious agalactia and their purpose'	Revised the scores given to some tests in Table 1, and added the loop-mediated isothermal amplification (LAMP) method
B.1.5. 'Molecular methods – detection of nucleic acids'	Updated and reformatted the Section using the tables of the primer/probe sequences and cycling parameters for the real-time and conventional PCRs to follow the chapter template. Renamed the subsection on micro-array analysis "isothermal molecular assays" and updated the text to include the LAMP method
C. Requirements for vaccines	Deleted the subsections on vaccines for <i>Mycoplasma mycoides</i> subsp. <i>capri</i> infection and <i>Mycoplasma capricolum</i> subsp. <i>capricolum</i> and <i>M. putrefaciens</i> as these mycoplasmas are not the focus of the chapter
References	Updated the list of references

Section/paragraph	Change
Appendices 1 to 6	Added six appendices justifying the choice of assays and their ratings given in Table 1

Chapter 3.10.2. 'Cysticercosis (including infection with *Taenia solium*)': the Commission reviewed chapter 3.10.2., which had been updated by disease experts.

The revised Chapter 3.10.2. 'Cysticercosis (including infection with *Taenia solium*)' is presented as [Annex 20](#) for first-round Member comment.

The main amendments include:

Section/paragraph	Change
B.1.9. 'Detection of circulating antigens'	Qualified the sentence on the poor sensitivity and specificity of the Ag ELISA by stating that the sensitivity increases to 90% and 100% for the detection of carcasses with one or more viable cysticerci, and more than 10 viable cysts, respectively. Also added that as the test is genus specific, areas co-endemic with for example <i>T. hydatigena</i> complicate the usability of the test, which is particularly the case for Latin America and (South-East) Asia
References	Updated the list of references

Chapters for Member comment with this report and their corresponding Annex numbers

	Annex	Chapter no.	Chapter title
1.	4	1.1.2.	Collection, submission and storage of diagnostic specimens
2.	5	1.1.3.	Transport of biological materials
3.	6	3.1.8.	Foot and mouth disease (infection with foot and mouth disease virus): appendices only
4.	7	3.1.11.	Leishmaniosis
5.	8	3.1.17.	Paratuberculosis
6.	9	3.3.3.	Avian infectious laryngotracheitis
7.	10	3.3.5.	Avian mycoplasmosis (<i>Mycoplasma gallisepticum</i> , <i>M. synoviae</i>)
8.	11	3.3.7.	Fowl typhoid and Pullorum disease
9.	12	3.4.4.	Bovine genital campylocacteriosis
10.	13	3.4.10.	Haemorrhagic septicaemia (<i>Pasteurella multocida</i> serotypes 6:b and 6:e)
11.	14	3.4.11.	Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis
12.	15	3.5.1.	Camelpox
13.	16	3.6.4.	Equine encephalomyelitis (Eastern, Western and Venezuelan)
14.	17	3.6.6.	Equine influenza (infection with equine influenza virus)
15.	18	3.6.10.	Glanders and melioidosis
16.	19	3.8.3.	Contagious agalactia
17.	20	3.10.2.	Cysticercosis (including infection with <i>Taenia solium</i>)

5.2. Chapter 3.9.1. 'African swine fever (infection with African swine fever virus)'

Following adoption on the vaccine section of Chapter 3.9.1. 'African swine fever (infection with African swine fever virus)' in May 2025, an expert group noticed that the introductory paragraph to Section B.2. 'Serological tests' still stated that no commercial vaccines for ASF are available. The Commission proposed the following editorial amendment be made to that paragraph:

Serological assays are the most commonly used diagnostic tests due to their simplicity, relatively low cost and the fact that they require little specialised equipment or facilities. ~~For ASF diagnosis, this is particularly relevant given that no commercial vaccine is available against ASFV, which means that the presence of anti-ASFV antibodies always indicates infection. Furthermore, anti-ASFV antibodies appear~~ Soon after infection or vaccination (7–10 days), anti-ASFV antibodies appear and persist for several months or even years. Domestic pigs and wild boar infected with virulent strains usually die before a specific antibody immune response is raised. In areas with a well established ASFV infection, where attenuated and low virulent virus isolates are also circulating, serological detection is crucial for identifying recovered and asymptotically infected animals. There are no fully neutralising antibodies.

5.3. Health for animals: request to develop a new chapter on the application of the 3Rs¹ principle in veterinary vaccine production and to include the 3Rs concept in disease-specific chapters

Advances in non-animal methods (NAMs) have enabled the wider implementation of the 3Rs principle, improving precision, reproducibility, and efficiency while reducing reliance on animal testing. Lack of global harmonisation limits their adoption, particularly for international pharmaceutical companies. Health for Animal requested the Commission to consider developing a new chapter in the *Terrestrial Manual* on the 3Rs principle in veterinary vaccine production, along with the progressive integration of 3Rs considerations into disease-specific chapters.

The Commission is aware that there is variability in the quality of veterinary vaccines and that it would have a sizable impact on some small vaccine manufacturers were WOA to insist on the use of NAMs. Nonetheless, the Commission felt it is worth stating that alternatives to animal testing exist and noted that a statement to this effect has already been added to the disease-specific chapters as they are updated. The Commission is also aware that the 3Rs principle should be applied to diagnostic procedures and reagent production.

The Commission designated a member to review all the chapters in Section 2.3; 'Veterinary vaccines' of the *Terrestrial Manual* with the aim of exploring the best way to address the multiple proposals for inclusion of information on veterinary vaccines. The Commission will decide the way forward at the February 2026 meeting following the report of this analysis.

5.4. Question from the pre-General Session webinar on Chapter 1.1.9. 'Tests for sterility and freedom from contamination of biological materials intended for veterinary use'

During the pre-General Session webinar held in April 2025, one Member asked if a list of pathogenic bacteria and non-pathogenic bacteria that can be found as contaminants in live viral vaccines of poultry (drinking water) could be included in Chapter 1.1.9. 'Tests for sterility and freedom from contamination of biological materials intended for veterinary use'. The Member did not send a follow-up email as requested by the President during the webinar. In the absence of more information from the Member, the Commission cannot address this request.

5.5. *Terrestrial Manual* status: update on chapters selected for the 2026/2027 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 2026/2027 (year last adopted in brackets)

- 1.1.4. Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities (2015)
- 2.1.2. Biotechnology advances in the diagnosis of infectious disease (2021)
- 2.1.3. Managing biorisk: examples of aligning risk management strategies with assessed biorisks (2014)
- 2.2.7. Validation of diagnostic tests for infectious diseases applicable to wildlife (2014)
- 2.3.1. The application of biotechnology to the development of veterinary vaccines (2010)
- 2.3.2. Minimum requirements for the organisation and management of a vaccine manufacturing facility (2016)
- 2.3.4. Minimum requirements for aseptic production in vaccine manufacture (2016)
- 3.1.7. Epizootic haemorrhagic disease (infection with epizootic haemorrhagic disease virus) (2021)
- 3.1.8. Foot and mouth disease (infection with foot and mouth disease virus) (2025)
- 3.1.10. Japanese encephalitis (2021)
- 3.1.14. Nagana: infections with salivarian trypanosomoses (excluding *Trypanosoma evansi* and *T. equiperdum*) (2021)
- 3.1.15. New World screwworm (*Cochliomyia hominivorax*) and Old World screwworm (*Chrysomya bezziana*) (2019)

¹ The 3Rs are the guiding principles for more ethical use of animals in product testing and scientific research and stand for: replace, reduce, refine.

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- 3.1.18. Q fever (2018)
 - 3.1.22. Surra in all species (*Trypanosoma evansi* infection) (2021)
 - 3.1.26. West Nile fever (2018)
 - 3.3.6. Duck virus hepatitis (2017)
 - 3.3.8. Infectious bursal disease (Gumboro disease) (2016)
 - 3.3.10. Newcastle disease (infection with Newcastle disease virus) (2021)
 - 3.4.2. Bovine babesiosis (2021)
 - 3.4.8. Contagious bovine pleuropneumonia (infection with *Mycoplasma mycoides* subsp. *mycoides*) (2021)
 - 3.5.2. Middle East respiratory syndrome (infection of dromedary camels with Middle East respiratory syndrome coronavirus) (2021)
 - 3.6.7. Equine piroplasmiasis (2021)
 - 3.6.9. Equine viral arteritis (2013)
 - 3.8.4. Contagious caprine pleuropneumonia (2021)
 - 3.8.7. Ovine epididymitis (*Brucella ovis*) (2015)
 - 3.8.8. Peste des petits ruminants (infection with small ruminant morbillivirus) (2021)
 - 3.8.10. Scrapie (2022)
 - 3.8.12. Sheep pox and goat pox (2024)
 - 3.9.8. Transmissible gastroenteritis (2008)
 - 3.10.4. Verocytotoxigenic *Escherichia coli* (2008)

6. WOA Reference Centres

6.1. Applications for WOA Reference Centre status

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

WOAH Reference Laboratory for Trichinellosis

Laboratory for Animal Health - ANSES (French Agency for Food, Environmental and Occupational Health & Safety)
Maisons-Alfort, FRANCE

Tel.: +33 (0)1 49 77 28 16

Email: isabelle.vallee@anses.fr

Designated expert: Dr Isabelle Vallee

WOAH Collaborating Centre for Antimicrobial Resistance in Terrestrial Animals

Animal and Plant Quarantine Agency, APQA (under the Ministry of Agriculture, Food and Rural Affairs, MAFRA)
177, Hyeoksin 8-ro, Gimcheon-si, Gyeongsangbuk-do, 39660, KOREA (REP. OF)

Tel.: +82-54-912-0738

Email: imsk0049@korea.kr

Website: <http://www.qia.go.kr>

Designated Contact Point: Dr Suk-Kyung Lim

Four applications were received from the Middle East region for designation as Reference Laboratories for foot and mouth disease (FMD), peste des petits ruminants (PPR), dourine, and surra. The Commission welcomed these initiatives and underlined the importance of strengthening expertise in the region. Nevertheless, the applications demonstrated a need to further develop technical expertise, engage in international collaboration, and strengthen specialisation in the respective diseases. In particular, the ability to develop diagnostic methods, produce and provide reagents, conduct agent characterisation, receive and analyse samples, carry out research, and provide training were identified as areas requiring improvement. The Commission was informed that some applicants are considering applying for a WOA Twinning Project, which was considered more appropriate at this stage of their development. The Commission recalled that applications are assessed against the [criteria](#) outlined in the [Terms of Reference](#) for Reference Laboratories, which require institutions to demonstrate the ability, capacity, and readiness to fulfil the functions of a WOA Reference Centre. The Commission recommended that the FMD Reference Laboratory candidate engage with the [WOAH/FAO FMD network](#). The applications were not accepted.

One application was received from the European region for designation as a Reference Laboratory for enzootic bovine leukosis. The Commission noted that the laboratory has participated in WOA capacity building activities, with progress observed in quality assurance and proficiency testing. However, the application fell short in several key areas: no evidence of training activities, limited disease-specific expertise of the nominated expert, and insufficient publication record in the disease area. Furthermore, the laboratory requires additional time to strengthen its international profile and to progress towards accreditation (ISO 17025 or equivalent). The Commission concluded that the application was premature and encouraged them to maintain their efforts and resubmit the application in the future when the issues identified have been addressed.

One application was received from the Asia and Pacific region for designation as a Reference Laboratory for equine influenza. The Commission recognised the important role the laboratory has played during the recent outbreak and looks forward to forthcoming publications. However, the Commission considered that stronger international engagement is required, including participation in proficiency testing schemes with other countries and closer collaboration with existing WOA Reference Laboratories. The laboratory is currently perceived as largely national in scope and would benefit from expanding its international activities and establishing training opportunities for neighbouring countries. The Commission concluded that the application was premature and should be reconsidered once additional capacity has been developed.

Finally, an application from the Americas region for designation as a Collaborating Centre for Biorisk Management was reviewed. The Commission noted that the applicant's expertise and experience were not sufficiently aligned with the requirements and expectations of a WOA Collaborating Centre. The Commission recommended strengthening scientific output in the area of biorisk management, increasing the number of specialised human resources, and incorporating additional experts in the field. The Commission also considered that the applicant should enhance collaboration with institutions in the region. It was further noted that a WOA Collaborating Centre for Laboratory Biorisk Management already exists in the region. The application was not accepted.

6.2. Changes of experts at WOA Reference Centres

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

Swine streptococcus

Prof. Zongfu Wu to replace Prof. Chengping Lu at the Branch of Swine Streptococcosis Diagnostic Laboratory of the Nanjing Agricultural University (NAU), CHINA (PEOPLE'S REP. OF)

Chronic wasting disease

Dr In-Soon Roh replace Dr Hyun-Joo Sohn at the Prion Disease Research Laboratory, Division of Foreign Animal Disease, Animal and Plant Quarantine Agency (QIA), Ministry of Agriculture, Food and Rural Affairs (MAFRA), KOREA (REP. OF)

Foot and mouth disease

Dr Su-Mi Kim to replace Dr Sang-Ho Cha at the Division of Foot and Mouth Disease, Animal and Plant Quarantine Agency (QIA), Ministry of Agriculture, Food and Rural Affairs, KOREA (REP. OF)

Contagious equine metritis

Dr Laura Florea to replace Dr Ian Mawhinney at the Animal and Plant Health Agency, UNITED KINGDOM

Mammalian tuberculosis

Dr Mark Andrew Chambers to replace Dr Jason Sawyer at the Animal and Plant Health Agency, UNITED KINGDOM

Leptospirosis

Ms Karen LeCount to replace Dr Matthew Erdman at the USDA APHIS Veterinary Services, Diagnostics and Biologics, UNITED STATES OF AMERICA

The Commission reviewed three additional nominations for change of expert for brucellosis (*Brucella abortus*, *B. melitensis* and *B. suis*), rabies and salmonellosis and based on the information provided found that the nominees did not fulfil the expectations of a WOA Expert.

6.3. Review of new and pending applications for laboratory twinning

As of September 2025, 100 projects have been completed, 15 projects are underway, and one project is waiting signature before starting. The completed projects have resulted in the designation of 20 new WOA Reference Laboratories and four new WOA Collaborating Centres.

The Commission supported the technical contents of the following four twinning proposals:

- **United Arab Emirates and Mongolia** on camel diseases;
- **Italy and Tajikistan** on quality management biosafety and biosecurity;
- **Italy and Kyrgyzstan** on quality management biosafety and biosecurity;

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- **Germany and Lithuania** on next generation, whole genome sequencing techniques and bioinformatics analyses of viruses and bacteria causing animal diseases important to international trade and human health.

6.4. Update on the first group of self-assessments of the relevant Centres 5-year work programme

At the February 2025 meeting, the Commission reviewed and accepted 59 self-assessments from terrestrial Collaborating Centres for which the 5-year designation concluded at the end of 2024. All Centres confirmed their intention to renew their designation.

At the September 2025 meeting, the Commission reviewed and accepted eight additional late self-assessments, accompanied by explanations for the delays. These Centres also confirmed their willingness to remain within the network. All these Collaborating Centres will be formally notified that their designation will be renewed and will be requested to submit a new 5-year workplan covering the period 2026–2030.

For the remaining four Collaborating Centres that reached the end of their 5-year designation in 2024 but that did not submit their self-assessment, a letter will be sent to the relevant Delegates, with the Collaborating Centres in copy, reminding them of Article 10 of the [Procedures for Designation](#), which refers to the performance criteria agreed by the Commissions in February 2018, including the failure to respond to requests from WOA Headquarters. Unless a satisfactory explanation is provided, the Commission will consider de-listing these Centres.

6.5. Update on the second group of self-assessments of the relevant Centres 5-year work programme

The Commission was informed that in the current year, only two Collaborating Centres will be concluding their 5-year designation (2021–2025). Requests for the submission of their 5-year self-assessments will be sent in the last quarter of 2025, for evaluation by the Commission at its February 2026 meeting.

The Commission noted that every 5 years there will be many self-assessments to review from those Centres that submitted their reports for the first time in 2020, following the introduction of the 5-year workplan procedure. As a result, the workload for the review of self-assessments will be significantly higher every 5 years, and considerably lower in the years in between. The Commission invited the Secretariat to consider this imbalance when drafting the agenda of the Commission's meetings.

6.6. Semi-automatic evaluation system for Collaborating Centre annual reports

Since 2023, a semi-automatic evaluation system has been in place for the review of annual reports submitted by the Reference Laboratories. This system assigns scores to each question and flags submissions that may indicate a performance risk. It helped to standardise evaluations and improve efficiency by directing attention and resources to reports with a higher likelihood of underperformance that require closer review by a member of the Commission.

Due to the effectiveness of this system, and with the same objectives in mind, the Commission decided at its February 2025 meeting to extend the semi-automated evaluation system to the annual reports of Collaborating Centres. The aim is to establish an effective digital mechanism able to detect potential underperformance with a high degree of sensitivity, while reducing the workload of the Commission. The system will categorise Collaborating Centres as low or high risk of underperformance. This approach also ensures that the number of reports that each member must evaluate can be reduced, while ensuring that every Collaborating Centre is reviewed at least once every 3 years.

In line with the criteria agreed by the Commission in September 2025, the essential elements of the Terms of Reference are those that all Centres must meet. Of the 12 questions currently included in the questionnaire, three (questions 4, 7 and 8) have been designated as essential.

The Commission agreed to implement the system for the review of the 2025 annual reports.

6.7. Videos of diagnostic techniques available on WOA website

Since February 2023, WOA Reference Laboratories have been invited to submit videos demonstrating diagnostic techniques related to their designated diseases. These videos complement the methods described in the *Terrestrial Manual* and are intended to support the practical understanding and implementation of international standards.

In 2024, specific submission criteria were established to ensure the quality and suitability of videos accepted into the repository. Following the February 2025 meeting, the first two videos were formally endorsed by the Commission and made available through the [WOAH website](#).

During this September 2025 meeting, the Commission also reviewed and accepted an additional video submitted by a Reference Laboratory on diagnostic tests for *Aethina tumida* and *Tropilaelaps* spp. infestations.

The Commission encourages all Reference Centres to continue contributing to this initiative by submitting videos illustrating diagnostic methods described in the *Terrestrial Manual*.

6.8. Annual reports of activities of WOA Reference Centres

6.8.1. Follow-up on under-performing Centres

The Commission reviewed the feedback received from 38 Reference Laboratories and nine Collaborating Centres that were identified as not fully complying with key performance criteria in their 2024 annual reports. In most cases, the justifications provided were considered acceptable and therefore endorsed by the Commission. For those responses that lacked sufficient justification or did not meet expectations, the institutions were placed on a watch list.

Following the review, 14 Reference Laboratories were placed on the watch list, three were suspended due to persistent non-compliance with the Terms of Reference (linked to accreditation constraints), and two withdrew their designation as WOA Reference Laboratories. Seventeen laboratories provided satisfactory explanations, and the Delegates of the respective countries will be notified accordingly.

Among the Collaborating Centres, four were placed on the watch list, three provided satisfactory explanations, and two did not respond. The Delegates of the concerned countries will likewise be informed.

The Commission expressed its appreciation to all Reference Laboratories and Collaborating Centres for their responses and acknowledged their continued commitment to advancing animal health. The Commission took noted that the Deputy Director General would send an acknowledgment letter to those Reference Laboratories.

6.8.2. How to evaluate Reference Laboratories that do not receive samples for multiple reasons independent of their capacity or willingness to accept the samples

When reviewing the annual reports of the Reference Laboratories, the Commission noted that factors beyond the laboratories' capacity limited the capacity of the Reference Laboratories to receive international sample for confirmatory diagnostics. Those factors include strict national legal framework, not receiving requests from abroad and other external limitations.

The Commission further observed that several Reference Laboratories demonstrated strong performance at the national level, but not at the international level. To assess the Reference Laboratories' international engagement, the Commission agreed to pay special attention during the 2025 annual reports assessment to the extent and quality of international activities reported by all WOA Reference Laboratories. In particular, the responses to Questions 1 and 10 of the annual report template.

Should a consistent pattern of limited international contribution be identified, the Commission agreed that a formal communication will be addressed to the Reference Laboratory and their respective WOA Delegate.

6.8.3. How to evaluate Reference Laboratories for non-listed diseases

The Commission identified 15 Reference Laboratories covering ten non-listed diseases or areas (including antimicrobial resistance). This review was prompted by the fact that 80% of these laboratories (12/15) had been flagged as potentially underperforming by the semi-automated evaluation system of annual reports. The Commission noted, however, that while 80% were identified as at risk, only 33% (5/15) were placed on the watch list, primarily due to prioritisation considerations linked to the diseases concerned.

The Commission further acknowledged that the current evaluation methodology is not fully adapted to Reference Laboratories working on non-listed diseases, as these laboratories often receive fewer samples or resources owing to epidemiological factors or institutional priorities. Nonetheless, the Commission underlined the continued strategic importance in maintaining international expertise in these diseases, emphasising the need to ensure sufficient capacity to respond effectively to any potential outbreaks.

It was therefore agreed that these Reference Laboratories would be reviewed individually during the Commission's February 2026 meeting.

6.9. Outcome of the satisfaction questionnaire from Collaborating Centres

To gain insight into the experiences and perspectives of WOAHA Collaborating Centres, the Commission developed a questionnaire to better understand their engagement, satisfaction, and the benefits of being part of the network. This initiative followed a similar survey conducted in 2023 for Reference Laboratories, which had provided valuable feedback on interactions with WOAHA and the Commission, as well as opportunities for improving existing processes.

The questionnaire, which focused on aspects not already covered in the annual report, was open from March to May 2025 covered aspects related to communication, procedures, and priorities. It was circulated to all Collaborating Centre contact points, and 45 responses were received from 76 experts (a 60% response rate), representing all five WOAHA regions. The questionnaire was structured into seven sections covering different dimensions of the Collaborating Centre system. Overall, the results reflected a high level of satisfaction among respondents.

The main benefits reported by Collaborating Centres included the international recognition of their institutes and experts, as well as the opportunity to connect with similar organisations. Respondents highlighted the need to simplify the annual report platform. Only half of the Collaborating Centres had been asked by WOAHA to contribute to revisions of the *Terrestrial Manual* over the past 5 years; those involved expressed great satisfaction with the process but also requested feedback on their contributions and stronger collaboration with the relevant Specialist Commissions.

The results further indicated the need for greater interaction between Collaborating Centres and their Delegates. Fewer than half reported having been consulted by their Delegates on draft chapters, though those who were involved described the experience positively and wished for more opportunities. A similar pattern was seen regarding representing WOAHA at international meetings: only about half of the respondents had been invited to do so, but all expressed strong satisfaction when given the opportunity. In general, the Collaborating Centres expressed a clear desire for stronger support and engagement from WOAHA, the Specialist Commissions, and their Delegates.

Experts also emphasised the importance of closer and more regular contact with the Commission and WOAHA staff, ideally through in-person exchanges, to strengthen networking, align efforts, and set joint goals.

The Commission considered this survey to be an important source of information to guide improvements in the functioning of Collaborating Centres. The results will be shared with all Reference Centres as feedback and are attached as [Annex 21](#) in this report.

6.10. Update on WOAHA Reference Centres networks

African swine fever

The ASF Reference Laboratory Network meets quarterly to harmonise and validate diagnostic tools, provide training and expertise to WOAHA Members, and share global data on ASF occurrence and virus genetics. The ASF Network actively monitors ASF vaccine development and use and provided technical input to the Commission on Section C. 'Requirements for vaccines' of the *Terrestrial Manual* [Chapter 3.9.1](#), adopted in May 2025. Dr Livio Heath, the expert head of WOAHA Reference Laboratory for ASF for South Africa and Co-Chair of the Network, chaired the [ad hoc Group](#) reviewing the draft guidelines on field evaluation and post-vaccination monitoring of ASF vaccines in July 2025. In addition, the network published a [manual on the protocols and guidelines for laboratory diagnosis of ASF](#) in June 2024, is updating [field testing guidelines/ PoC manual](#) to reflect the latest developments, and plans to launch a genomic platform in 2026 to enhance surveillance and research capacities.

Peste des petits ruminants

As of July 2025, the PPR Reference Laboratory Network comprises 25 laboratories, with a Secretariat led by CIRAD² (current chair), Pirbright, CAHEC³, ICAR-NIVEDI⁴, AU-PANVAC⁵, and the WOAHA PPR Secretariat, and with participation from IAEA⁶ since February 2025. Activities in 2024–2025 included the annual workshop, a series of "LABinars" focused on Africa and molecular diagnostics, annual training and proficiency testing organised by CIRAD, and contributions to the WOAHA Reference Centre Network Meeting in May 2025.

2 CIRAD: le Centre de coopération internationale en recherche agronomique pour le développement (French Agricultural Research Centre for International Development)
3 CAHEC: China Animal Health and Epidemiology Centre
4 ICAR-NIVEDI: ICAR-NIVEDI, Indian Council of Agricultural Research–National Institute of Veterinary Epidemiology and Disease Informatics
5 AU-PANVAC: African Union Pan African Veterinary Vaccine Centre
6 IAEA: International Atomic Energy Agency

Upcoming activities include additional training workshops, LABinars, and the annual newsletter, as well as participation in the PPR Global Research and Expertise Network (GREN) meeting hosted by CAHEC in November 2025 in Qingdao, China (People's Rep. of).

Rabies

The WOAHP Rabies Reference Laboratory Network (RABLAB) currently consists of 14 laboratories and meets bimonthly. RABLAB continues to strengthen global coordination for rabies diagnostics and surveillance, supporting WOAHP Members through laboratory capacity-building projects, technical guidance, and contributions to One Health rabies control initiatives.

Three United Against Rabies Country Partnership Programmes are now underway: Malawi supported by Onderstepoort Veterinary Institute, South Africa; and Guinea and Liberia supported by CDC⁷.

Additional country/region-focused activities are ongoing to support laboratory capacity building and national planning in North Africa, building of regional laboratory networks in Southern Africa and South Asia, and training for Oral Rabies Vaccination of dogs in Southern Africa.

In 2025 the '[WOAHP Rabies Reference Laboratory Network's \(RABLAB\) overview of LFD tests for field application](#)' was published. This document summarises RABLAB's current knowledge of commercially available Lateral Flow Devices/Point of Care tests and provides practical guidance on the appropriate use of these as a complementary tool within national rabies surveillance systems.

FMD

The WOAHP/FAO⁸ FMD Reference Laboratory Network actively supported regional roadmap and SEACFMD⁹ meetings in 2025, providing updates on the global situation and vaccine matching. Key highlights included participation in the GF-TADs¹⁰ Middle East FMD/PPR Regional Roadmap workshop in April 2025, the 27th SEACFMD meeting in August 2025, and the WOAHP Reference Centre Network Meeting in May 2025, where discussions focused on the Nagoya Protocol and the genomic platform OpenFMD. The annual meeting of the Network will take place in Istanbul in October 2025, alongside the Global FMD Research Alliance meeting.

6.11. Rinderpest

The Commission was updated on the implementation of rinderpest post-eradication activities. WOAHP continues to work in partnership with FAO to reduce the rinderpest virus containing material (RVCM) 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-WOAHP designated Rinderpest Holding Facilities (RHF) Category A, in addition to the reduction of RVCM held by WOAHP Members in unauthorised institutes. In this regard, after destroying all RVCM except for vaccine seed, CIRAD, France, had the Category A designation removed and retains only the Category B designation, which allows the laboratory to continue to hold vaccine seed on behalf of FAO for global use. Similarly, The Pirbright Institute, United Kingdom, has announced that all RVCM except vaccine seed for diagnostic positive controls will be destroyed before the end of 2025. An upcoming outbreak modelling study will inform the ideal size of the global rinderpest vaccine reserve.

Regarding specific RHF, the RVCM held by the Plum Island Animal Disease Center, New York, United States of America, will be moved to the new Foreign Disease Diagnostic Laboratory at the National Bio and Agro-Defense Facility in Kansas. The FAO-WOAHP Rinderpest Secretariat will organise a site inspection by March 2026. The National Institute of High Security Animal Diseases (NIHSAD), India, applied to be designated as a RHF in Categories A & B and a site inspection took place in March 2025. NIHSAD was designated as a RHF Category A in May 2025. The Category B designation was left pending on the corrective actions raised in the inspection report being addressed by the end of the year. In May 2026, the designation period of all eight currently designated RHF comes to an end. Hence, at its meeting in November 2025, the FAO-WOAHP Joint Advisory Committee will review the implementation of the recommendations and corrective actions prescribed during the respective inspections and advise FAO and WOAHP on the extension of the designation of each RHF.

There are four Members keeping RVCM outside FAO-WOAHP designated RHF: Iran, Russia, South Africa, and Türkiye. WOAHP urges these Members to comply with the provisions of Resolution No. 21/2017 by destroying these RVCM or sequestering them at an FAO-WOAHP designated RHF. The Federal Centre for Animal Health "FGBI

7 CDC: Centers for Disease Control and Prevention (of the United States of America)

8 FAO: Food and Agriculture Organization of the United Nations

9 SEACFMD: South-East Asia and China Foot and Mouth Disease Campaign

10 GF-TADs: Global Framework for the Progressive Control of Transboundary Animal Diseases

ARRIAH", Russia, applied in September 2025 to be designated as an RHF Category B. The application is being evaluated for completeness by the FAO-WOAH Rinderpest Secretariat before being assessed by the FAO-WOAH Joint Advisory Committee.

6.12. Update on the animal welfare network

The Chair of the WOAH Collaborating Centre for Animal Welfare Network, Dr Luigi Iannetti and the Vice-Chair, Dr Carolyn Guy, presented the network's 5-year Work Plan (2025–2029).

The Plan sets the strategic framework for the network, which is governed by a Steering Committee composed of representatives from the four WOAH Animal Welfare Collaborating Centres and their partners. Leadership rotates on a 2-year basis, with the Vice-Chair assuming the role of Chair at the end of each term. Through this comprehensive working plan, the WOAH Collaborating Centre Network for Animal Welfare aims to achieve measurable advances in animal health, supporting global resilience and progress in veterinary science and welfare.

The strategic objectives of the Network are to enhance interdisciplinary cooperation, expand capacity building, promote adoption of international standards and support WOAH's global programmes in surveillance and risk management, through a One Health/ One Welfare approach.

The planned activities for the Network that are set out in the plan include expert meetings and training, the development and dissemination of technical resources, collaborative research and outreach and communication of the Network.

The importance of monitoring and evaluation activities such as the regular review and progress reports is reflected in the plan, to ensure that progress and impact is tracked.

6.13. Change of the legal address but not facilities of a Collaborating Centre and three Reference Laboratories hosted by the Federal State-Financed Institution "Federal Centre for Animal Health" (FGBI "ARRIAH") in Vladimir, Russia

The Federal Service for Veterinary and Phytosanitary Surveillance (the Rosselkhoz nadzor) informed the Headquarters that due to internal reorganisation, the legal address of the Federal State Financed Institution "Federal Center for Animal Health" (FGBI "ARRIAH"), subordinate to the Rosselkhoz nadzor, will change from Vladimir to Moscow. This adjustment is purely administrative and will not affect the physical location or operations of FGBI "ARRIAH," which will remain in Vladimir.

The Commission noted the change. The FGBI ARRIAH hosts the WOAH Collaborating Centre for Diagnosis and Control of Viral Animal Diseases in Eastern Europe, Central Asia and Transcaucasia, and the WOAH Regional Reference Laboratories for FMD, for avian influenza and for Newcastle disease.

6.14. SOP¹¹ for testing of thermotolerant PPR vaccine

PPR poses a major economic and production threat. Control efforts rely on two live attenuated vaccines, Nigeria 75/1 (line II) and Sungri/96 (line IV), which provide long-lasting immunity but require a cold chain, creating logistical challenges in the field. To address this, several techniques have been developed for production of thermotolerant vaccines using improved formulation of freeze-drying stabilisers and the lyophilisation processes.

In 2017, the workshop organised by FAO/WOAH PPR-Secretariat with the support of the Global Alliance for Livestock Veterinary Medicines (GALVmed) recommended establishing criteria for assessing thermotolerant vaccines independently to the technique used to improve PPR vaccine stability. AU-PANVAC, with support from GIZ¹² and the African Union, has since undertaken work to develop acceptance criteria to ensure their safe and effective use. The data from the study on the Criteria for assessing the thermotolerance of PPR have been presented during various PPR-GREN and PPR vaccines manufacturers meetings. These data were recently published in the peer review journal (*Viruses* [2025], **17**, 1151; <https://doi.org/10.3390/v17091151>).

The proposed criteria require the evaluation of thermotolerant PPR vaccine titre after 5 days' incubation at 40°C: (1) the vaccine should maintain the minimum titre of $10^{2.5}$ TCID₅₀/ml for vaccine dose, and (2) the titre loss must not exceed 1 log₁₀.

11 SOP: Standard Operating Procedure

12 GIZ: La Deutsche Gesellschaft für Internationale Zusammenarbeit

13 TCID: tissue culture infective dose

The PPR Network members proposed that the *Terrestrial Manual* should incorporate criteria for assessing the acceptability of thermotolerant vaccine batches in Section C.2.2.4 or C.2.3.3 on batch release. The PPR chapter was included in the list of chapters to be updated in the cycle 2026/2027.

On the other hand, it was recommended that a detailed step-by-step SOP for testing thermotolerant PPR vaccines should be prepared and maintained through available platforms such as the WOAHP Reference Laboratory Network website. The SOP could be revised depending on the new developments.

The Commission was requested to review and provide recommendations on the criteria and the SOP for testing thermotolerant PPR vaccines. The Commission agreed on the incorporation of the criteria for assessing thermotolerant of PPR vaccines in the *Terrestrial Manual* chapter 3.8.8 (Section C.2.2.4 or C.2.3.3 on batch release).

7. Ad hoc Groups

7.1. Ad hoc Group on Replacement of the International Standard Bovine and avian Tuberculin (ISBT)

At its February 2025 meeting, the Commission had requested a detailed update on the experiments conducted to replace the ISBT and highlight the areas of concern in the experiment. Based on this, the Chair of the *ad hoc* Group provided an overview of the experiments completed so far for the replacement of the ISBT.

The Commission noted that since the beginning of the project in 2015, there have been significant challenges in evaluating guinea pig assays to determine the potency of replacement candidates.

The Chair explained that, since the potency of Candidate B is estimated by comparing it to the current ISBT standard, several hypotheses were tested through a series of experiments conducted by UKHSA¹⁴ between 2022 and 2025 for potency evaluation of Candidate B. These hypotheses included possible degradation, precipitation of the ISBT used, and the low pathogenicity of the *Mycobacterium bovis* AN5 strain, which could have influenced the overall experimental outcomes. Additionally, the interferon gamma release assay experiments performed in 2024 indicated that Candidate B and the current ISBT were aligned in terms of antigenicity from the immunological point of view.

The Commission was informed that the latest guinea pig assays had continued to show inconsistent results, despite the Group's best efforts to fine-tune the protocol. These results were attributed to a weak dose–response, high variability in skin test results, varied immune responses among the animals, and a consistent lack of reproducibility across studies and between assays.

The Group recommended conducting one final round of blinded guinea pig assays using the same protocol, but this time through a private tuberculin manufacturing company. Given their extensive experience in successfully performing these assays at a commercial scale, the Group expected that this method may yield more consistent results. The Commission acknowledged the Group's concern and agreed to be notified electronically by the Secretariat on the results of the final assay.

The Commission was also informed that if the upcoming experiments were successful, there would be no further trials and Candidate B would be proposed for identifying as a new standard. However, if it fails, the Group has proposed that WOAHP should continue its efforts to identify a new candidate and relaunch the call for candidates, while simultaneously developing a roadmap to advance the work required to validate molecularly defined tuberculin (MDT) as a potential replacement for ISBT, considering that the current ISBT stock is expected to last only for 2 to 5 years.

The Commission highlighted that while MDT shows promise as an alternative to traditional biological PPD, its sensitivity seems to be lower albeit with a higher specificity compared to PPD in naturally infected animals. The Chair acknowledged these concerns and proposed that the roadmap for the development of MDT would also include these considerations, as well as the regulatory, scientific, and field work required in different epidemiological settings to establish MDT as an alternative to ISBT in the future.

Regarding the ISAT, the Commission was updated on the ongoing assessment of two candidate products being evaluated for their feasibility as potential ISAT standards. The process includes pre-evaluation, freeze-drying, and post-assessment of the freeze-dried products, which is scheduled to be completed in the first quarter of 2026. The Chair of the *ad hoc* Group explained that, unlike ISBT, the ISAT assays use heat-killed antigens and are therefore not expected to exhibit the same level of variability in experimental outcomes. The Commission would be updated on the results of these assessments at its February 2026 meeting.

14 UKHSA: UK Health Security Agency

8. International standardisation/harmonisation

8.1. Virtual biobank

The Commission was updated on the Virtual Biobank project, led by the WOAHP Collaborating Centre for Veterinary Biologicals Biobank, hosted by the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZSLER), Italy.

The expert reminded the Commission that the project has two main objectives: the development of an online catalogue and the establishment of the Biobank Management System (BMS), a dedicated software platform designed to register and manage data related to biological material. At present, the system is undergoing bug testing and performance improvements. The next steps will include a second round of testing with Italian national veterinary institutes, followed by an international trial phase involving selected WOAHP Reference Laboratories with varying IT capacities.

A technical issue discussed previously concerned the controlled taxonomy file for parasites and fungi, with similar challenges identified for bacteria, where only genus and species classifications were available rather than the full taxonomy. A temporary solution has been proposed, allowing the manual inclusion of taxonomic names until a more comprehensive system is developed.

The expert highlighted that, while some IT issues remain to be addressed, the system is ready for deployment and initial validation. Broader international testing and subsequent refinements will ensure the system's gradual and evolutionary improvement in the years ahead.

The Commission congratulated the project team, noting that the system appears highly impressive and that earlier feedback had been effectively integrated. Members expressed enthusiasm for its future application and enquired about the expected timeline for access by smaller Reference Laboratories. The current plan foresees a second round of testing with Italian institutes in October 2025, a third round with four to five international Reference Centres in the first semester of 2026, and the release of the Virtual Biobank and BMS to all WOAHP Reference Centres by late 2026 or early 2027.

8.2. Update on the Register for WOAHP-approved diagnostic kits

The Commission was updated on the decision to review the WOAHP Register of Diagnostic Kits. To guide this process, an *ad hoc* Group of experts will be established to assess the added value, feasibility and framework of a new registration system, ensuring its long-term sustainability and positive impact. The *ad hoc* Group will also explore the setting of minimum validation criteria for reliable registration, to facilitate accessibility for all Members regardless of their regulatory capacity. In addition, the experts are expected to define the roles and responsibilities of WOAHP Reference Laboratories and Collaborating Centres in diagnostic kit recognition procedures, as well as in the development of validation training and guidance packages. The *ad hoc* Group will also provide input on mechanisms that could contribute to greater regulatory harmonisation.

The Commission was invited to appoint members, either in an active or oversight capacity, to follow the work of the *ad hoc* Group, and approved a 1-year extension of the freeze on all activities of the Secretariat for Diagnostic Test Kits until May 2027, to allow sufficient time for the completion of this revamp.

8.3. Procedure for designating WOAHP Standards Reagents

According to the Terms of Reference for WOAHP Reference Laboratories: [Reference Laboratories should] "store and distribute to national laboratories biological reference products and any other reagents used in the diagnosis and control of the designated pathogens or diseases". In fulfilment of this mandate, WOAHP-approved reference reagents play a critical role in ensuring the availability of high-quality diagnostic materials to WOAHP Members. These reagents provide a standardised platform that enables Reference Laboratories to share validated biological materials, thereby fostering global cooperation and enhancing laboratory diagnostic capacities worldwide.

Furthermore, as outlined in WOAHP *Terrestrial Manual* Chapter 1.1.6. 'Validation of diagnostic assays for infectious diseases of terrestrial animals', Sections B.1.4 and B.4.4 – reference reagents are essential for quality control, proficiency testing, and harmonisation purposes.

To support this, a procedure has been developed to govern the management of WOAHP-approved reference reagents for the diagnosis of infectious animal diseases, covering submission of requests for inclusion in the list of approved International Standard Reagents, the decision-making process regarding their acceptance, and the ongoing maintenance of their status. Additional follow-up actions were identified, including the need to add guidance on batch

consistency to the existing guidelines, to propose a checklist to assist in the evaluation of applicants, and to share with experts the applicant template.

The Commission welcomed the progress made in reactivating the list of WOAHA-approved reference reagents, underlining the importance of this initiative for global animal health. The Commission expressed their satisfaction at seeing this work advance and reaffirmed the value of reference reagents in strengthening international diagnostic capacity. The Commission agreed to review the latest version of the template and the guidelines following the meeting, with the shared objective of finalising the [procedure](#) and having it operational by the end of the year.

9. Follow-up from the General Session

9.1. Update and excerpt from the Final Report

The Commission was informed that the presentation to the Assembly during the General Session in May 2025 was very well received by the Delegates. Two chapters had received technical comments from the floor. Those comments had been addressed in this September Commission meeting. For Chapter 3.1.8. 'Foot and mouth disease (infection with foot and mouth disease virus)', the technical comments on criteria used to define vaccine potency will be discussed at the forthcoming meeting of the WOAHA/FAO FMD Reference Laboratory Network, which will be held in Istanbul, Türkiye from 22 to 24 October 2025. The Delegate of the Member will be informed of the outcome of discussions. For Chapter 3.9.1. 'African swine fever (infection with African swine fever virus)', the Commission felt that the comments had been addressed in the draft chapter. The Delegate of the Member will be informed of the Commission's position.

9.2. Animal Health Forum: Vaccine and vaccination

The Commission took note of the outcomes of the [2025 Animal Health Forum](#), the [Technical Item: Veterinary Vaccines and Vaccination: from science to action – reflections for change](#), and the adoption of [Resolution No. 29](#). In its discussion, the Commission highlighted several points of relevance to its mandate.

The Commission agreed to review relevant *Terrestrial Manual* chapters to ensure provisions for new vaccine technologies are not unnecessarily restrictive, while maintaining requirements for safety and efficacy. It recommended engaging vaccine producers to identify concrete proposals for revision and underlined the importance of WOAHA facilitating dialogue to avoid standards acting as a barrier to innovation.

In addition, in response to Recommendation 8 and of Resolution No. 29, the Commission proposed that experts are requested to include a sub-section in part C of the disease-specific chapters of the *Terrestrial Manual* on post-vaccination monitoring with particular emphasis on correlates of protection (e.g. differentiating infected from vaccinated animals: DIVA), to evaluate safety, efficiency, field-level effectiveness.

The Commission highlighted that Reference Centres may provide expertise to Members on vaccine quality and strategies but are not mandated to certify vaccines. It recommended that WOAHA formally communicate this to Reference Centres, with reference to Resolution No. 29.

9.2.1. TPPs (target product profile)

The Commission welcomed the development of a standard template for Target Product Profiles (TPPs), drawing on existing models, to provide practical guidance while remaining aligned with WOAHA standards.

Finally, the Commission underlined the importance of dialogue between public and private sectors, ensuring engagement of vaccine producers included both international and local producers.

9.2.2. List of priority animal diseases for which new or improved vaccines could achieve the greatest reductions in antimicrobial use

The Commission reviewed the terms of reference for a planned *ad hoc* group on updating the evidence-based list of priority animal diseases for which vaccines could achieve the greatest reductions in antimicrobial use. This work will build on lists WOAHA developed in 2015 and 2018 through two previous *ad hoc* expert groups. Using a multi-criteria decision framework, the *ad hoc* Group will rank candidate diseases in three production sectors: pigs, chickens, and cichlids. For the top priority diseases for which a new or improved vaccine is needed, the commission recommends the drafting of target product profiles (TPPs) that set out desired vaccine characteristics to strategically direct vaccine R&D and production investment. The Commission agreed with the proposal to convene experts from diverse sectors (animal health, food producing industry, vaccine R&D and manufacturing and veterinary products regulators) and regions to produce the TPPs and a

communication, dissemination and exploitation plan for 2026–2030. The Commission proposes repeating this exercise for large and small ruminants in a second phase.

The Commission was invited to appoint a member, either in an active or oversight capacity, to follow the work of the *ad hoc* Group.

10. Conferences, Workshops, Meetings

10.1. Report WOAHA Reference Centre Network Meeting and implementation plan – May 2025

In May 2025, WOAHA hosted a joint meeting of Reference Centre networks at its headquarters. Experts on ASF, FMD, PPR, rabies, OFFLU—the WOAHA-FAO network of expertise on animal influenza—and wildlife health convened to strengthen collaboration and facilitate knowledge exchange and sharing between various Reference Centre networks, identify areas for collaboration across the networks by finding synergies and provide recommendations and guidance in overcoming shared cross-cutting technical and operational challenges for enhancing the diagnosis and surveillance of priority animal diseases.

The Commission was updated on the outcomes of the meeting and presented with an implementation plan for its comments to follow up on the recommendations identified from the meeting to address the following key cross-cutting issues faced by networks.

Recommendations:

a. ISO 35001

WOAHA considered the requirement for directing laboratories to obtain ISO 35001 accreditation, which describes key components of a biorisk management system intended to protect containment laboratory personnel, visitors, contractors and the public from accidental release of pathogenic material.

The Commission noted that obtaining ISO 35001 accreditation is very challenging and involves significant costs and efforts, which impacts the whole institution. The accreditation may not be relevant to laboratories handling certain pathogens, e.g. bee diseases, and is prone to variations in interpretation. The Commission agreed to encourage laboratories to follow a system that aligns with ISO 35001, but did not agree to make it a mandated requirement; they did, however, agree to include a question in the annual report template to gather information from the Reference Laboratories on their use of this ISO standard.

b. Annual report evaluation

The Reference Centre experts who participated in the meeting suggested that the Headquarters send an acknowledgement letter to those Reference Centres with an excellent level of performance, as demonstrated in their annual reports, for better recognition and institutional visibility.

The Biological Standards Commission Secretariat informed that such letters are already sent to these Reference Centres and showed a copy of an example letter to the Commission. The Commission asked the Biological Standards Commission Secretariat to copy the Delegate and, if possible, the Director of the institute, the next time these letters are sent.

c. Nagoya Protocol implementation

WOAHA and its Members support the implementation of the Nagoya Protocol through the development of technical guidance, pathogen emergency access protocols and national legislation. These efforts aim to strengthen disease surveillance, control and vaccine innovation.

The Commission agreed with the meeting recommendation to create a Nagoya task force to follow up on the actions identified in the meeting. The FMD network, which has published a report on the impact of Nagoya protocol, will lead the task force and involve suitable experts from other disease networks and in collaboration with tripartite (FAO-WHO-WOAHA) focal points working on this activity.

d. Genomic platforms fit-for-purpose

Sharing genetic material is currently hindered by inconsistent nomenclature, fragmented access to multiple databases, a lack of harmonised standards and high costs of designing and maintaining the platforms. Experts explored adapting existing platforms—such as OpenFMD—tailored to meet their respective network needs.

The Commission agreed with the meeting recommendation to create a group of experts from networks interested in genomic platforms, with ASF and FMD chairs as Chairs. The Commission asked to also involve the Collaborating Centre on Genome Monitoring of Swine Viral Disease, USA in this expert group.

e. Point-of-care tests for priority animal diseases

Point-of-care tests (POCT) offer rapid, on-site results that can guide immediate decision-making. Network experts will support the development of POCT for priority animal diseases, collaborating with the WOAHA Biological Standards Commission to integrate validation requirements in the *Terrestrial Manual*.

The Commission agreed with the meeting recommendation to ask individual Reference Centre networks to add discussion of POCT on the agenda of their regular meetings for follow up on the recommendation.

f. Availability and sharing of reagents

Reference Centres are responsible for producing and distributing quality-assured diagnostic reagents. Networks will make their reagents available from the WOAHA-approved reference reagents and coordinate with the Biobank project to align efforts.

The Commission was informed that the ASF network will propose a template for listing and sharing of reference reagents, and this template will be used by other networks. An internal shared database will be created for all the networks to list the available reference reagents. The Biological Standards Commission Secretariat was advised to give a short presentation on reference reagents to Reference Centre network meetings.

g. Sequence data sharing for network activities and public databases

Experts recommended establishing mechanisms for specimen and data sharing to accelerate research and development. A checklist will help ensure data, shared in standards, meets FAIR principles: Findable, Accessible, Interoperable and Reusable.

The Commission was informed that each network will discuss at their regular meetings, the benefits of sequence data sharing for network activities and prompt submission to public databases. The Commission commented that there is a need to standardise the format or structure that could be used by laboratories for sequence sharing and advised the Secretariat of the networks to collect information on lists of sequences shared in the Reference Centre annual report.

h. Capacity building and knowledge sharing

WOAHA was encouraged to integrate training videos in its *Terrestrial* and *Aquatic Manuals* and continue to promote One Health synergies between veterinary and public health laboratories. Experts also proposed establishing Reference Laboratories for common diagnostic methods such as ELISA and PCR.

The Commission was informed that each network will add this topic to the agenda of their regular meetings to identify the most common diagnostic methods used and videos available and discuss with Biological Standards Commission Secretariat for submission to integrate on the WOAHA website.

i. Proficiency testing

One of the core responsibilities of Reference Laboratories is to participate in and coordinate diagnostic proficiency testing (PT) to ensure diagnostic quality and consistency. Experts proposed resource-efficient PT strategies aligned with the *Terrestrial Manual* and recommended publishing a list of PT schemes, organisers and indicative costs on the WOAHA website.

The Commission agreed with the meeting recommendation to create a task force led by the PPR network Secretariat to develop a concept note detailing strategies for organising resource-efficient PTs aligned with the provisions of the *Terrestrial Manual*. The Commission commented that information on the use of ISO 17043 accreditation status for proficiency testing organisers could be collected in the annual report, noting that accreditation to this standard will remain as desirable but not mandatory. The Commission also agreed to publish a list of available PT schemes for various diseases in the websites of the disease networks.

j. Shipment of samples and reagents

Shipping challenges undermine laboratory operations in endemic settings. Experts urged WOAAH to support national capacity building for safe, compliant shipment of infectious materials, including training on International Air Traffic Association (IATA) regulations and updated guidance.

The Commission was informed that the recommendation will be linked with the PT recommendation and followed by all network Secretariats. The Commission was informed that the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) accepts fast track registration for all WOAAH Reference Centres, allowing transfer of samples from CITES-listed species hence supporting Members in managing cross-border sample movement for these species. The Biological Standards Commission Secretariat will be informed of the two-step registration process in this regard, which could be shared with all Reference Centres.

k. Laboratory twinning programme

The Commission was informed of the recommendations of the laboratory twinning programme evaluation and future plans that will be followed up by the laboratory twinning manager.

l. Engaging Members and regional networks

The Reference Centre networks aim to involve more laboratories from low-and middle-income Members and address their capacity needs. Strengthening ties with regional networks like GF-TADs was also recommended. Members are encouraged to actively engage with Reference Centres, even those beyond their own regions.

The Commission was updated that each global disease-specific network will make a list of regional and sub-regional networks and map the ongoing activities for collaboration.

m. Network outputs

The Commission was updated that network Secretariat will develop an internal share point with sections and subsections for all networks to share various network outputs like annual workshops, newsletters, website, webinar, international meetings, regional/sub-regional disease specific work plans, etc.

10.2. WOAAH seminar held during the WAVLD Symposium in Calgary, Canada, June 2025

As a side event at International Symposium of the World Association of Veterinary Laboratory Diagnosticians (ISWAVLD), WOAAH held an international design thinking workshop to identify solutions for improving the sustainability of laboratories. The event was organised under the BioPrevail banner, in collaboration with Global Affairs Canada (GAC) and the Global Health Security Fund, with funding provided by GAC.

Eighteen international experts attended, including laboratory specialists, policy experts from the security and health sectors, and representatives from both the public and private sectors. The workshop produced concrete ideas to address challenges negatively affecting laboratory sustainability. Discussions also informed the development of a design sprint, which was scheduled to launch later in the summer.

11. Matters of interest for consideration

11.1. WOAAH–WHO collaboration on biological risk management along the pathogen value chain

The premise for this work was highlighted in the Biosafety Research Roadmap—a collaboration between WOAAH, WHO, and Chatham House. One of the resulting articles noted:

“Consideration needs to be given to the wider context of pathogen research in which laboratories exist. Many activities outside the laboratory environment are subject to very little oversight and governance. Attending to the risks presented by the whole chain of activity, from sample collection in the field to disposal of waste, is important.”

Chatham House, 2023

Recognising this need, WOAHA has begun discussions with relevant stakeholders, including WHO, to explore the development of a One Health approach to biological risk management across the entire pathogen value chain—from survey design and sample collection, to transport, processing, testing, and waste disposal.

The WOAHA Biological Standards Commission has endorsed this approach, and WOAHA will seek funds to support its implementation.

11.2. BioPrevail: Design sprints and the broader initiative

BioPrevail is a collaboration between WOAHA, Global Affairs Canada, the UK International Biosecurity Programme, and the Global Health Security Fund. It is an innovation ecosystem designed to identify and scale innovative solutions that strengthen the sustainability of diagnostic high-containment laboratories.

In August 2025, a design sprint was launched to address sustainability challenges in three domains: facility design, equipment maintenance, and waste management. Sixty-six applications were received, of which 44 advanced to the judging phase. Nine teams were selected, against strict criteria, for further development. These teams will receive mentoring on customer discovery, intellectual property, pitching, and other skills, before presenting their ideas to potential investors and stakeholders at a showcase event in December 2025.

This initiative will also be presented alongside the WOAHA Biosafety Roadmap at the Global Conference on Biological Threat Reduction (28–30 October 2025).

11.3. VICH activities

The Commission received a report on key VICH¹⁵ events held in The Netherlands, in November 2024, the 43rd Steering Committee meeting, the 17th Forum, and the [7th Public Conference](#). The Conference, held every 5 years, focused on strategies to support the global availability of safe and effective veterinary medicinal products through regulatory convergence, international cooperation, and efficient registration processes.

The Commission was also informed that the VICH Biologicals Expert Working Group has drafted the first version of the guideline titled “*Test for the Presence of Extraneous Viruses in Veterinary Vaccines*”. WOAHA requested that the final version be shared during the consultation phase to ensure its consideration in the relevant Section of the *Terrestrial Manual* (Section 2.3. ‘Veterinary vaccines’). The next Steering Committee and Forum meetings will take place in November 2025.

11.4. Laboratory Focal Points meeting: Biorisk management

As part of the FIRABioT project, WOAHA has supported Kenya and Malawi in developing biological risk management guidance tailored to field operations and veterinary laboratories, respectively. In both initiatives, WOAHA’s expertise network—including reference laboratories, collaborating centres, and PVS experts—played a critical role in reviewing and refining the draft documents.

During Kenya’s process, a need was identified for the Commission to lead efforts in establishing consensus definitions for field biosafety and field biosecurity. WOAHA standards were applied throughout these activities, as well as in related training sessions on surveillance and diagnostics.

Several expertise gaps were noted across the continent, particularly in French-speaking countries. These gaps have necessitated the involvement of human health professionals in building capacity among animal health professionals, and the engagement of institutions outside WOAHA’s expertise network to support diagnostics training.

WOAHA also conducted the third cycle of its regional training for national veterinary laboratory focal points in Africa. These sessions helped identify priority areas for reference laboratories and led to the proposal of criteria for their designation.

15 VICH: International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products

Following discussions, the Commission recognised the need to explore the feasibility of initiating twinning projects focused on generic topics—rather than disease-specific ones—as a strategic approach to addressing cross-cutting expertise gaps in countries.

12. Strategic and Creative thinking

The Commission agreed to have a link to equivalence studies and any other validation studies on POCTs on the Reference Laboratory webpage. As an initial step, the ASF and rabies POCT information documents, developed by the respective networks, will be made available through that link.

The Commission also agreed to ask the experts when requesting their annual reports, if there is any pressing modification they wished to propose to their *Terrestrial Manual* chapter that would then be reviewed in isolation. For example, in the face of outbreaks due to a novel strain, the experts might wish to include details of an appropriate PCR assay. This would ensure that the needs of WOAHA Members are met in a more effective manner.

These proposals illustrate that the Commission is prepared to embrace new technologies that are fit for purpose and contribute to WOAHA's response in the face of evolving disease patterns.

.../Annexes

Annex 1. Adopted Agenda

MEETING OF THE WOAHP BIOLOGICAL STANDARDS COMMISSION

Paris, 8–12 September 2025

1. Welcome

- 1.1. Director General
- 1.2. Joint meeting with all Specialist Commissions
 - 1.2.1. WOAHP 8th Strategic Plan
 - 1.2.2. Recommendations of the Governance Review Committee
 - 1.2.3. Animal Health Forum
 - 1.2.4. Global Conference on Biological Threat Reduction
- 1.3. Transparency of commenting – phased approach

2. Adoption of the agenda

3. Liaison with other Specialist Commissions

- 3.1. Terrestrial Animal Health Standards Commission
 - 3.1.1. Glossary term: laboratory
 - 3.1.2. Advice to the TAHSC regarding a Member comment on draft Code chapter on Crimean–Congo haemorrhagic fever
 - 3.1.3. Bureau meeting
- 3.2. Scientific Commission for Animal Diseases
 - 3.2.1. Case definitions: infection with *Chlamydia abortus*, anthrax and Aujeszky's disease
 - 3.2.2. Guidelines for African swine fever vaccines: field evaluation and post-vaccination monitoring

4. Work Programme

5. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals

- 5.1. Review of draft chapters and their endorsement for circulation for first-round Member comment
- 5.2. Chapter 3.9.1. 'African swine fever (infection with African swine fever virus)'
- 5.3. Health for animals: request to develop a new chapter on the application of the 3Rs principle in veterinary vaccine production and to include the 3Rs concept in disease-specific chapters
- 5.4. Question from the pre-General Session webinar on Chapter 1.1.9. 'Tests for sterility and freedom from contamination of biological materials intended for veterinary use'
- 5.5. *Terrestrial Manual* status: update on chapters selected for the 2026/2027 review cycle

6. WOAHP Reference Centres

- 6.1. Applications for WOAHP Reference Centre status
- 6.2. Changes of experts at WOAHP Reference Centres
- 6.3. Review of new and pending applications for laboratory twinning
- 6.4. Update on the first group of self-assessments of the relevant Centres 5-year work programme
- 6.5. Update on the second group of self-assessments of the relevant Centres 5-year work programme

-
- 6.6. Semi-automatic evaluation system for Collaborating Centre annual reports
 - 6.7. Videos of diagnostic techniques available on WOAHA website
 - 6.8. Annual reports of activities of WOAHA Reference Centres
 - 6.8.1. Follow-up on under-performing Centres
 - 6.8.2. How to evaluate Reference Laboratories that do not receive samples for multiple reasons independent of their capacity or willingness to accept the samples
 - 6.8.3. How to evaluate Reference Laboratories for non-listed diseases
 - 6.9. Outcome of the satisfaction questionnaire from Collaborating Centres
 - 6.10. Update on WOAHA Reference Centres networks
 - 6.11. Rinderpest
 - 6.12. Update on the animal welfare network
 - 6.13. Change of the legal address but not facilities of a Collaborating Centre and three Reference Laboratories hosted by the Federal State-Financed Institution “Federal Centre for Animal Health” (FGBI “ARRIAH”) in Vladimir, Russia
 - 6.14. SOP for testing of thermotolerant PPR vaccine
 - 7. Ad hoc Groups**
 - 7.1. Ad hoc Group on Replacement of the International Standard Bovine and avian Tuberculin (ISBT)
 - 8. International standardisation/harmonisation**
 - 8.1. Virtual biobank
 - 8.2. Update on the Register for WOAHA-approved diagnostic kits
 - 8.3. Procedure for designating WOAHA Standards Reagents
 - 9. Follow-up from the General Session**
 - 9.1. Update and excerpt from the Final Report
 - 9.2. Animal Health Forum: Vaccine and vaccination
 - 9.2.1. TPPs (target product profile)
 - 9.2.2. List of priority animal diseases for which new or improved vaccines could achieve the greatest reductions in antimicrobial use
 - 10. Conferences, Workshops, Meetings**
 - 10.1. Report WOAHA Reference Centre Network Meeting and implementation plan – May 2025
 - 10.2. WOAHA seminar held during the WAVLD Symposium in Calgary, Canada, June 2025
 - 11. Matters of interest for consideration**
 - 11.1. WOAHA–WHO collaboration on biological risk management along the pathogen value chain
 - 11.2. BioPrevail: Design sprints and the broader initiative
 - 11.3. VICH activities
 - 11.4. Laboratory Focal Points meeting: Biorisk management
 - 12. Strategic and Creative thinking**

Annex 2. List of Participants

MEETING OF THE BIOLOGICAL STANDARDS COMMISSION

Paris, 8–12 September 2025

MEMBERS OF THE COMMISSION

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(President)
Professor of Virology
CNRA/LIRED
Abidjan
CÔTE D'IVOIRE

Prof. Ann Cullinane
(Vice-President)
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Prof. Chris Oura
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Dr Mariana Delgado
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Science Department

Annex 3. Work Programme for the WOA Biological Standards Commission

MEETING OF THE BIOLOGICAL STANDARDS COMMISSION

Paris, 8–12 September 2025

Subject	Issue	Status and Action
Updating the <i>Terrestrial Manual</i>	1) Circulate the chapters approved by the Biological Standards Commission to Members for first-round comment	Deadline for comments 31 December 2025
	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) Ask Reference Centres to provide links to suitable instructional videos to be added to the WOA website. Videos to be reviewed by the Commission when the chapter is up for review (optional)	On-going
	4) Review new developments in diseases causing significant global impacts (e.g. avian influenza, African swine fever) and prioritise those chapters	On-going
	5) Start the process of addressing the request to have access to the previous versions and evolution of the <i>Terrestrial Manual</i> as done with the <i>Terrestrial Code Proposal</i> : - At the chapter level go to the different versions - Have "Previous editions of the <i>Terrestrial Manual</i>" with the whole version of the year X. With a watermark to make it clear that it is not the current version	On-hold
	6) Review pathogen taxonomy, including virus classification, and subsequent changes to chapter titles	On-going
	7) Harmonise the subheadings throughout the chapters (e.g. serology vs antibody detection)	On-going
	8) Pre-General Session Webinar: presentation of chapters proposed for adoption during the 2025 General Session	Accomplished
	9) Repository of equivalence studies for point of care tests as a new tab on the Biological Standards Commission webpage and a link in the chapters when POC are mentioned (starting point: ASF and rabies)	On-going
	10) Thermotolerance of PPR vaccines assessment protocol	On-going
	11) Include section on DIVA and post-vaccination monitoring in Section C of disease-specific chapters	On-going
	12) Review the structure and contents of all vaccination chapters	Biological Standards Commission member
	13) Develop new chapters or sections: - Post-vaccination monitoring 3Rs for vaccines and diagnostics	On-hold (pending point 12 and 14)
	14) Develop decision tree for establishing new chapters	February 2026
	15) Change ToRs of the Consultant Editor to focus on critical elements of the draft chapters that must be reviewed	February 2026

Subject	Issue	Status and Action
	16) Discuss the purpose of the <i>Terrestrial Manual</i> in view of expanding chapter lengths and the number of chapters	February 2026
	17) As chapters are revised, give instructions when difficulties are encountered in matrices when tests are used for international trade. Biological Standards Commission will engage with other Commissions to seek their advice on improvement in the <i>Terrestrial Manual</i> in this regard	On-going
Collaborating Centres	1) Evaluate the feedback from those Centres that completed 5 years and assess the current relevance of the scope of their activities for renewal	On-going
	2) Invite those Collaborating Centres that have successfully completed their 5-year designation to submit a new 5-year work plan for 2026–2030	On-going
	3) Evaluate the 5-year work plan of the CC starting a new term	November 2025
	4) Increase visibility of current Centres: ask to submit maximum of five bullet points to be added to their website entry under the title “How can we help?”	On-going/accomplished
	5) 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
	6) Finalise the questionnaire to gather feedback from the Collaborating Centres on their experiences being a WOAHA Collaborating Centre, and send it to the Centres	Accomplished
	7) Prepare 1 pager summarising the results of the Collaborating Centre satisfaction questionnaire and share the results with the Reference Centres network	February 2026
	8) Highlight the need to close the gap between WOAHA Delegates and their Reference Centres when reviewing chapters circulated for revision	Include in the agenda of the Regional Commission conferences – On-going
	9) Implement the semi-automatic evaluation system for annual reports	Before the meeting to review the annual reports (March-April)
Reference Laboratories	1) Put under-performing laboratories on watch list and monitor their performance	On-going
	2) Assess the annual reports for 2025	March-April 2026
	3) WOAHA to explore how to extract the detailed data from the annual reports to be used to map the international activities of the Reference Laboratories	On hold
	4) Explore a way to highlight the achievements of highly functioning laboratories	February 2026
	5) Ensure that the letters addressed to well performing Reference Laboratories are also directed to their Delegate	On-going
Reference Centre Networks	1) Follow up with the six Reference Centre networks (ASF, PPR, FMD, OFFLU, rabies and welfare)	On-going
	2) Organise the joint Reference Laboratory network meeting	Accomplished

Subject	Issue	Status and Action
	3) Inform the Biological Standards Commission about the outcomes of the recommendations from the Reference Laboratory network meeting	On-going
Standardisation/ Harmonisation	1) Project to extend the list of WOAHA-approved reference reagents	Accomplished
	a) Start the process of collecting applications for WOAHA approved reagents from Reference Laboratories	On-going
	2) Project to develop Replacement International Standard Bovine and Avian Tuberculin: finalise report and propose for adoption	On-going
	3) Update on the discussion carried by the <i>ad hoc</i> Group on the decision on maintaining or not the procedure of assessment on WOAHA approval for diagnostic kits	On-going
Ad hoc Groups	1) Contribute on the review on the <i>Terrestrial Code</i> Chapter 4.7. Collection and processing of bovine, small ruminant and porcine semen	On-hold (check with Code Secretariat)
Projects	1) Veterinary Biobanking (project)	On-going
	2) BioPrevail: an initiative designed to foster sustainable laboratory solutions	On-going
Conferences, Workshops and Meetings with participation by Biological Standards Commission Members	1) ISWAVLD WOAHA Seminar: develop a theme and programme and speakers. June 2025 in Canada	Accomplished
	2) General Session 2025: Animal health forum on Vaccine & vaccination	Accomplished
	3) Participation in the Regional Commission Conferences	Accomplished
Performance	1) Engage with the ongoing processes around performance of Reference Laboratories	On-going
Develop laboratory standards for emerging diseases	1) Discuss the <i>Terrestrial Code</i> chapter once adopted and consider introducing a corresponding chapter for the <i>Terrestrial Manual</i>	After May 2026
Case definitions	1) Follow up the implementation of the SOPs for case definitions	On-going

MEETING OF THE BIOLOGICAL STANDARDS COMMISSION

Paris, 8–12 September 2025

CHAPTER 1.1.2.

**COLLECTION, SUBMISSION AND STORAGE
OF DIAGNOSTIC SPECIMENS**

INTRODUCTION

Laboratory investigation of animal disease is critically dependent on the quality and appropriateness of the specimens collected and submitted for analysis. This chapter sets out the general standards involved in specimen collection, submission, and storage. The individual disease chapters in this Terrestrial Manual provide specific information on appropriate specimens needed to test for particular pathogens, biomarkers, or toxins. Sampling may be from individual animals, from animal populations, or from the environment for a variety of purposes, such as disease diagnosis, disease surveillance, health certification, and monitoring of treatment or vaccination responses. To ~~provide~~ ensure scientifically and statistically valid results the specimens collected must be ~~appropriate~~ suitable for the intended purpose, and adequate in quality, volume, and number for the proposed testing. Additionally, the animals and tissues sampled must ~~be appropriately representative of the condition being investigated~~ accurately represent the condition under investigation.

Specimens must be collected using appropriate biosafety and containment measures in order to prevent contamination of the environment, animal handlers, and individuals doing the sampling as well as to prevent cross-contamination of the specimens themselves. Regulations should be followed, including for the sampling of protected animals. Care should additionally be taken to avoid undue stress or injury to the animal and physical danger to those handling the animal. Biological materials should be packaged to rigorously control for leakage, and then labelled with strict adherence to the applicable regulations guiding their transport as outlined in Chapter 1.1.3 Transport of biological materials.

A. COLLECTION OF SAMPLES SPECIMENS

1. General considerations

Careful consideration must be given to the collection, containment, and storage of the specimens, including biosafety measures that must be in place to prevent contamination of the environment or exposure of other animals and humans to potentially infectious materials (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*). For information on transport of specimens see Chapter 1.1.3 *Transport of biological materials*.

The reliability of the diagnostic testing is critically dependent on the specimen(s) being appropriate, of high quality, and representative of the disease ~~process or condition~~ being investigated. Prior to sampling, consideration must be given to the type of specimen(s) needed, including the purpose of the testing and the test technologies to be used. The volume or quantity of specimen must be sufficient to perform initial testing, to perform any subsequent confirmatory testing and to provide sufficient residual specimen for referral or archival purposes.

The purposes of testing will be aligned with the purposes for which tests are validated, as listed in Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*, ~~namely:~~

- i) ~~Demonstration of freedom from infection in a defined population.~~
- ii) ~~Certification of freedom from infection or presence of the agent in individual animals or their products for trade/movement purposes.~~
- iii) ~~Eradication of disease or elimination of infection from defined populations.~~
- iv) ~~Confirmatory diagnosis of suspect or clinical cases.~~
- v) ~~Estimation of prevalence of infection or exposure to facilitate risk analysis.~~
- vi) ~~Determination of immune status of individual animals or populations (post vaccination).~~

Epidemiologically appropriate sampling plans should be developed prior to collection of specimens, as described in Section B and Appendix 1.1.2.1. These will specify the number of animals or other sampling units to be sampled.

Specimens ~~must~~should be collected according to a sound knowledge of the epidemiology and pathogenesis of the disease under investigation, or the disease syndrome to be diagnosed, by a trained and competent person, taking into consideration animal welfare, relevant regulations and ethics. In the absence of sufficient knowledge (for instance, unusual host species), the specimen collection strategy has to be adapted to maximise the chance of detecting the causative agent(s). This will lead to the sampling of tissues or fluids most likely to contain the infectious agent or evidence of the infection. Considerations will include the tissue predilection(s) or target organ, the duration and site of infection in each tissue type and the duration and route of shedding, or the time frame in which evidence of past infection, such as an antibody response, can be detected reliably by the tests to be deployed. Host ectoparasites and environmental specimens can also be considered for pathogen detection. These considerations will also indicate The method(s) of collection should also be considered when selecting the specimen type to be used. In many herd or flock-based disease investigations, it is beneficial to collect specimens from a healthy cohort for comparative epidemiological or baseline testing (e.g. case-control and cohort approaches for diagnostic testing) and for validation purposes.

Where chemical euthanasia, sedation or anaesthesia is required for animal restraint, the impact on animal welfare and scope for the chemical to influence ~~on~~ the test result (e.g. toxicology testing) must be considered. Some laboratory tests are not compatible with specific blood anticoagulants and tissue preservatives, such as heparin, formalin, dry ice (exposure of the test sample to elevated levels of CO₂), or even freezing. While it is critical to collect specimens as aseptically as possible, equal care must be taken to avoid contamination with detergents and antiseptic treatments used to clean the collection site on the animal, as these agents may interfere with the laboratory test procedures. Procedures requiring tissue culture of pathogens, as well as many molecular-based tests, can be negatively affected by chemicals or detergents commonly used in the manufacture or preparation of collection tools (e.g. chemicals used in manufacture of some types of swabs and detergents used in cleaning glassware).

Specific information on diagnostic test methodologies and the recommended specimens, preservatives, and specimen handling procedures can be found in the individual *Terrestrial Manual* disease chapters or through direct consultation with the laboratory that will be performing the required testing. Procedures for collection and submission of specimens are available from most diagnostic laboratories, including national and international authorities, where the information is frequently accessible via the specific laboratory's web page. The WOAHP web page provides contact information for all WOAHP reference laboratories¹.

It is critical not only to collect the most diagnostically appropriate specimens, but to also inform the laboratory of the associated disease epidemiology, aiding in order for the laboratory scientists to in assigning the most appropriate tests or panels of tests. Epidemiological information to be submitted with specimens is outlined in Section C of this chapter.

Where investigating diseases of unknown cause multiple different specimens that represent the different stages of the disease progression in an animal or the population of animals (e.g. the pre-clinical, early clinical, active clinical, chronically affected and convalescent phases) should be collected. Appropriate control specimens can also be collected whenever metagenomics and metaxonomics (microbiome exploration) are considered. Epidemiological considerations for sampling are particularly critical for diagnosing population-related diseases, as would occur with beehives, flocks, and herds. Epidemiological principles used for sampling are further introduced in Section B of this chapter. Investigation of pathogens transmitted at the livestock/wildlife/human interface may require a multi-pronged and interdisciplinary (One Health) approach.

1 <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>

Specimens can be collected ante-mortem or post-mortem and specimen ownership aspects need to be clarified before sampling. Consideration related to transport of specimens and biosecurity are described in Chapter 1.1.3 Transport of biological materials and Chapter 1.1.4 Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities. compliance with national and international regulations is needed for sampling and specimen transfer. Sampling of wild species is frequently subject to specific permits from wildlife authorities and should be aligned with existing wildlife management plans. Consideration of sampling of endangered wildlife protected by national or international regulations may require expert consultation, a risk analysis or a cost-benefit analysis and should consider practicalities of access to remote locations, specific expertise required, lack of basic data, carcasses degradation (scavengers or autolysis). When possible, sampling invasiveness should be reduced and non-invasive alternatives should be sought, especially with wild animals (Schilling *et al.*, 2022). Consultation with an appropriate veterinary diagnostic laboratory with expertise in wildlife is critical for success and may include field training (WOAH, 2025).

Specific considerations regarding different specimen types are as outlined below.

2. Blood

Whole blood samples may be collected for ~~haematology, clinical chemistry, toxicology,~~ direct examination for bacteria or parasites, PCR testing, untargeted metagenomic diagnostics or immunological testing, or for culture for bacteria or viruses. In addition, these specimens could be used for haematology, clinical chemistry or toxicology. Dependent on testing needs, whole blood, blood cells, and/or plasma samples can be obtained from whole blood collected into appropriate anticoagulants. In selecting the anticoagulant to be used the collector must be aware of the laboratory tests, including PCR-based diagnostics, clinical chemistry, and toxicology, which may be negatively affected by the presence of specific anticoagulants or preservatives. Specific disease chapters in this *Terrestrial Manual* provide guidance for individual tests and sample requirements. To be effective anticoagulants require that the collected blood be thoroughly mixed with the chosen anticoagulant during or immediately following its sampling from the animal.

To obtain serum, whole blood is collected without anticoagulants and the clot is allowed to contract at ambient temperature protected from extremes of heat and cold for periods that may range from a few hours to ~~overnight~~ several hours, while extended periods, e.g. overnight, should be avoided. Clear serum can be decanted or collected by pipette following physical removal of the clot, ideally following gentle centrifugation to separate cell components from the serum. In the absence of a centrifuge, separation of the clot can be facilitated by tilting the freshly collected blood tube at an approximate 45 degree angle until the clot has retracted, “ringing” the clot with a sterile rod or pipette to separate the clot from the tube surface, and then removing the clot with forceps. The results of serological testing can be compromised by the quality of the sample and bacterial contamination. ~~Bacterial contamination and red blood cell debris in serum samples can produce false positive reactions in agglutination-type assays. Serological assays can be negatively impacted by haemolysis in the serum sample. Microbial contamination and haemolysis are significant concerns especially when obtaining blood and serum samples from post-mortem animals.~~ Frequent causes of haemolysed serum and plasma include exposure to excessive temperatures or time delays prior to separating sera from the red blood cells, blood collection using a needle of too small gauge, or failure to remove the needle when transferring the blood sample from the collection syringe.

Whole blood should be collected aseptically, typically by venipuncture of the live animal. Depending on the animal and sampling situation jugular, caudal, brachial, cephalic, mammary veins or the vena cava may be used. Specific techniques for sampling small laboratory animals have been reviewed (Anon, 1993; Hem *et al.*, 1998). Care should be taken to collect and dispense blood samples as gently as possible to prevent damage to red blood cells, which causes haemolysis. Blood and sera are typically shipped and stored cool (or frozen in the case of sera) in non-breakable vials, tubes, or bottles; however for some laboratory tests that require viable peripheral blood mononuclear cells, the blood must be packaged, transported and stored so as to prevent exposure to temperature extremes. For some tests, aliquots of specimens can be dried onto a piece of untreated, or specifically treated commercial filter paper designed for stabilised sample transport and storage. However, in humid environments, the use of desiccants may additionally be required.

3. Faeces

Faeces can be collected freshly voided or preferably directly from the rectum/cloaca for tests such as culture for microorganisms, parasite examination, or faecal occult blood determination; or can be collected for culture and molecular-based diagnostics from the rectum/cloaca using cotton, dacron, or gauze-tipped swabs, dependent on the volume of sample required by the specific test methodology. Samples collected on swabs should be kept moist by placing them in the transport media recommended for use with the specific test to be performed, which may range from sterile saline to culture media containing antimicrobials or stabilisers. Faecal specimens should be kept chilled (e.g. refrigerated at 4°C or on ice) and tested ~~as soon after collection as possible preferably within 24–48 hours~~ to minimise the negative impacts on test results caused by death of the targeted microorganism, bacterial overgrowth or hatching of parasite eggs. Double-packaging of faecal samples in screw cap or sealable containers that are subsequently contained within sealed plastic bags will help prevent cross-contamination of samples and associated packaging materials. Faeces contained only in rectal exam gloves, plastic bags, or rubber-stoppered

tubes are unsuitable as they are very frequently comprised by bacterial growth with gas production that can rupture plastic bags, displace stoppers, and allow leakage of the specimen.

4. Urine

Urine sampling can be a non-invasive or minimally invasive method for detecting pathogens and physiological markers useful for surveillance of pathogens, renal function, systemic infections, and environmental toxins. It can provide crucial diagnostic information when blood or tissue sampling is logistically difficult or ethically undesirable. Urine samples can be collected during outbreaks, when non-invasive sampling is preferred for animal welfare or safety reasons, in longitudinal monitoring or from freshly deceased animals. Collection methods include spontaneous urination, manual expression, cystocentesis, catheterisation, absorbent material or surface swabbing. Samples can be kept refrigerated (for 24 hours), frozen, or conserved in RNA-stabiliser or other preservative.

45. Epithelium

Epithelial tissue in the form of biopsies or skin-scrapings; swabs of oral, nasal, pharyngeal, and gastrointestinal surfaces, as well as plucked hair or wool can be used variously for direct examinations or laboratory tests to identify surface parasites such as mites and lice, fungal, bacterial or viral infections, allergic reactions, and neoplasia. The specimens should be collected aseptically and preserved as specified for the intended test(s). Deep skin-scrapings obtained using the edge of a scalpel blade are useful for burrowing mites. Feather tips have been validated for use in the detection of viral antigen ~~for Marek's disease, and used as a sample for or~~ molecular detection of ~~additional~~ some avian diseases. Epithelial tissues, particularly those associated with vesicular lesions and collected into viral transport media, can be critical in the laboratory diagnosis of specific viral infections such as foot and mouth disease.

56. Ocular sampling

The surface of the eye can be sampled by swabbing or ocular scraping, ensuring that cells rather than mucopurulent discharge or lacrimal fluids are collected for testing. Specimens from the conjunctiva are typically collected by holding the palpebra apart and gently swabbing the surface of the eye with a cotton, dacron, or gauze swab that has been pre-moistened with sterile saline or equivalent media. Such swabs should be kept moist in saline or transport media specifically recommended for use with the testing to be performed. Biopsies from the third eyelid of sheep have been used as a lymphoid-rich tissue for prion detection. Aqueous humour serology is useful for the detection and measurement of antibodies and other substances related to eye infections.

67. Sampling the reproductive tract

Preputial and vaginal wash fluids and swabs of the cervix and urethra can be used as specimens for investigation of reproductive disease. The swabs should be kept moist following collection by placing in the recommended volume of transport media required by the laboratory test, typically sterile saline or specified culture media. Semen specimens are typically obtained using an artificial vagina or by extrusion of the penis and artificial stimulation. Avoid contamination of the specimen with antiseptic or detergent solutions used to prepare the animal/site for sampling.

78. Nasal discharge, saliva, and vesicular fluids

Secretions can be collected directly or with swabs into a vial or tube, or can be collected using swabs. Vesicular fluids provide a highly concentrated source of pathogens for diagnostic testing, and can be collected from unruptured vesicles using a sterile needle and syringe, and immediately transferred to a securely sealed vial or tube. Specifically developed sampling tools, such as probang cups, can be used for collecting cellular material and mucus from the pharynx of livestock. Cotton ropes that animals are allowed to mouth and chew ~~have been validated for use in collecting saliva specimens from domestic swine or food items discarded by the animals under investigation can be used to collect saliva specimens where applicable.~~

89. Milk

Milk can be collected from individual animals or from bulk milk in tanks pooled from multiple animals in a herd. The teat(s) used for sample collection should be cleaned, and any detergent thoroughly rinsed off before collection of the specimen. In collecting milk from individual teats, the initial stream must be discarded and only the subsequent streams sampled. The method of preservation prior to testing varies with the requirements of the test; in some cases it will be critical to avoid freezing or addition of chemical preservatives. The individual disease chapters of this *Terrestrial Manual* or the advice of the testing laboratory should be consulted for appropriate specimen handling and preservation recommendations.

910. Tissues collected at necropsy

The decision to conduct a post-mortem examination should be undertaken by qualified veterinarians or pathologists carefully considering human health and other risks. Necropsies should only be conducted by these trained individuals, although paraveterinary staff may be trained to conduct post-mortem examinations for specific purposes. Paraveterinary staff may be trained by veterinarians to conduct post-mortem examinations for specific purposes. Importantly, the purpose of the necropsy is not only to collect specimens but to make informed observations regarding the pathology of the condition. Such observations are an important adjunct to epidemiological and clinical observations in the comprehensive veterinary investigation of the case or outbreak. It is useful for veterinary authorities to retain specialist veterinary pathologists to lead post-mortem investigations in important cases. Where this expertise is managed from a veterinary laboratory the methods employed should be formally described in the laboratory's Quality Assurance Manual and the capability should be recognised in the laboratory's scope of accreditation. Detailed procedures for conducting post-mortem examinations and tissue collection are available in most pathology textbooks, and are additionally provided in many of the web-page accessible national laboratory testing guidelines. Specimens that are critical for the laboratory investigation of listed diseases are included in the chapters of the *Terrestrial Manual* relating to each disease.

Whether the necropsy is performed in a designated laboratory facility or in the field, appropriate biosafety and containment procedures should be followed to ensure operator safety and to provide non-contaminated and useful ~~tissues-samples~~ for testing as well as to protect the environment and other animals from potential exposure to pathogens. As a minimum requirement, the collector(s) must wear personal protective equipment that protects the skin and mucous membranes and that can be discarded or decontaminated. Specific information on biosecurity of wildlife necropsy is available (for instance, Wildlife Health Australia 2018). All remaining tissues or carcass parts and fluids should be contained and treated with an appropriate disinfectant or destruction method, and the immediate environment should be thoroughly disinfected.

~~Dependent~~ Depending on the suspected disease, condition of the carcass and facilities available for necropsies post-mortem specimens can be collected from one or multiple organs and tissues (including blood clots) and submitted to the laboratory as either fresh (no preservative) or preserved specimens for further laboratory testing. The process of carcass autolysis can destroy diagnostically relevant tissues and infectious agents and so should be considered prior to collecting and submitting post-mortem specimens.

For fresh specimens, particular attention must be paid to their handling and storage to avoid autolysis and overgrowth by bacterial and fungal contaminants. Ideally, freshly collected specimens are kept at a constant cool temperature from collection until processing for testing. Where such a cold chain cannot be provided fresh specimens for some test procedures can be collected into fluids such as ethylene glycol that transport medium that inhibits the growth of secondary organisms. This may, however, not be sufficient to maintain adequate quality of tissues susceptible to fast autolysis. Where such strategies are compatible with the subsequent test methods the option is mentioned in the relevant chapters of the *Terrestrial Manual* for each disease.

Preservation of post-mortem specimens is most frequently achieved by collection into formalin solution. This treatment, however, does not permit the testing of such specimens using culture or certain molecular methods. Where such chemical fixative is supplied to pathology staff by laboratories or competent authorities they must ensure adequate training in health and safety aspects of the use of such chemicals and training in compliance with regulations relating to the transport of such chemicals.

4011. Environment and feed

Environmental sampling may be of litter, bedding, water from troughs and drinkers, or feed which has been exposed to urine, faeces, and/or saliva of affected animals, or swabbed surfaces of facilities, ventilation ducts, drains or feed containers. If specialised equipment is available circulating air may be sampled. Environmental sampling of water, soil, and vegetation can provide valuable insights into pathogen presence and environmental contamination, particularly in ecosystems where direct animal sampling is difficult. Screening for environmental DNA (eDNA) is increasingly used to detect species presence and track pathogen circulation non-invasively. Proper collection requires sterile techniques, avoidance of cross-contamination, and the use of preservation methods appropriate to the target.

12. Arthropod insects

Ectoparasites are a valuable method for monitoring and diagnosing vector-borne pathogens, especially when direct sampling of animal hosts is not feasible. They can complement surveillance programmes and act as a proxy through xenosurveillance. Ectoparasites are collected during outbreaks of vector-borne diseases or in areas where host animals are difficult to capture or sample, and for proactive vector surveillance in endemic or high-risk areas. Targeted vectors include ticks, mites, lice, fleas, mosquitoes, and flies, including tsetse flies. Collection methods include direct skin removal, trapping, baiting, stick traps and aspirators. Preservation and transport methods depend

on the intended analysis, some ectoparasites such as ticks can be transported live to the laboratory whereas others require ethanol, DNA-stabilisers or cold chains.

11.3. Honey bees

Adult moribund or recently dead bees can be collected in the vicinity of colonies. Live bees can be killed by freezing either shortly after collection or following receipt at the laboratory. Brood specimens are typically collected by removing a piece of brood comb showing abnormalities and including dead or discoloured brood followed by wrapping in a paper towel or newspaper rather than in foil or wax paper in order to help prevent microbial overgrowth. Alternately, diseased cells in a comb may be sampled using a toothpick or equivalent. A sticky board can be used to collect hive debris, including trapping of mobile parasites. More information on the specimens that need to be collected can be found in the disease-specific chapters of this *Terrestrial Manual* related to bees.

B. EPIDEMIOLOGICAL APPROACHES TO SAMPLING

To provide scientifically and statistically valid results specimens must be appropriate for the intended purpose for the proposed investigation, and adequate in quality, volume, and number. The range of purposes for which investigations supported by laboratory testing may be conducted ~~have been outlined in Section A above~~ can be found in chapter 1.1.6.

For the purpose of laboratory testing to establish a diagnosis, it is important to sample animals that are either clinically affected, or suspected on good evidence to be infected or, for serology, to have been infected. Specimens that are most likely to give highest sensitivity and specificity to the investigation should be collected. In general, the stage of infection, as well as the route and duration of shedding, will determine the appropriate animal(s), stage of clinical disease, timeline for sampling, and tissue or anatomical site for sampling. In some cases, internal and/or external parasites may be useful in the confirmation of the condition. These criteria will be addressed through an understanding of the pathogenesis of the disease for known conditions and on a hypothesis of the pathogenesis of the diseases for conditions of unknown aetiology.

To detect evidence of infection in line with the other five purposes of testing as listed in ~~Section A above~~ chapter 1.1.6, the sampling should be done within the context of a surveillance programme. The criteria for the design and implementation of effective surveillance are described in Chapter 1.4 *Animal health surveillance* of the *Terrestrial Animal Health Code (Terrestrial Code)*. Identification of animals for sampling in surveillance programmes may be targeted (risk-based) or random. Risk-based sampling based on epidemiological knowledge of the infection under study or on epidemiological observations of the population under study is intended to result in the most likely detection of infected individuals. For wild animal testing, the lack of validated tests for numerous wild species is frequently a limitation (Chapter 2.2.7 *Validation of diagnostic tests for infectious diseases applicable to wildlife*).

Inferences on the status of a population such as estimation of prevalence, immune status or disease freedom should be based on random sampling. Random sampling ensures that the animals sampled are representative of the population, within the practical constraints imposed by different environments and production systems. Additionally, random sampling allows extrapolation of the findings of the study to the overall population (with an appropriate confidence interval). The epidemiological principles for sample size estimation are addressed in Appendix 1.1.2.1.

The specific requirements for surveillance to demonstrate freedom from disease/infection, and the associated sampling requirements, are addressed in detail in Article 1.4.6 of Chapter 1.4 of the *Terrestrial Code*.

Sampling and laboratory testing may also be used in support of epidemiologically based diagnostics and studies such as case control, structured longitudinal, and cohort studies (Fosgate & Cohen, 2008; Mann, 2003; Pfeiffer, 2010). The number and selection of the animals to be sampled and the nature of the specimens will be part of the study design. When wildlife population size is unknown, sampling strategies must rely on non-probabilistic or adaptive approaches. Convenience sampling, targeted sampling in high-risk areas or seasons, and opportunistic sampling (e.g. hunter-harvested or roadkill animals) are commonly used. Repeated sampling over time and space can help build a more representative picture. In such contexts, it is essential to document sampling effort, location, and selection criteria to support data interpretation and reduce bias. Where possible, combining multiple data sources (e.g. direct observation, camera traps, environmental DNA) can help infer population structure and pathogen dynamics.

C. INFORMATION TO BE SENT WITH SPECIMENS

Individual specimens must be clearly identified using appropriate methods. Marking instruments ~~should or labels must~~ be able to withstand the conditions of transport, storage and use, i.e. being wet or frozen. ~~Use of an indelible marking pen is required.~~ Pencil may rub off containers. Labels attached to plastic may fall off when stored at -70°C .

Information regarding the location and contact information of the submitter and the premises or area sampled, the case information and associated epidemiological information, as detailed below, should always accompany the specimens to the laboratory. Such documentation should be placed in a plastic envelope on the outside of the shipping container so as to be available for reference during transport and should also be duplicated inside the shipping container between the secondary and the outer packaging (see also Chapter 1.1.3 *Transport of biological materials*). It would be advisable to contact the receiving laboratory to obtain an appropriate submission form and other relevant shipping and handling information.

Necessary information includes:

1. Location and contact information

- i) Name and address of owner/occupier of the animal owner and/or the sampled premises and the geolocation (latitude and longitude, if available), where disease occurred, ~~with appropriate contact information (telephone number, e-mail address).~~ Note that this information can be sensitive in case of protected wild animal species.
- ii) Name, postal and e-mail address, telephone and fax numbers of the ~~sender~~ submitter.

The level of urgency associated with the testing should be indicated, and if appropriate the laboratory should be contacted in advance of receipt of the specimen.

2. Case information

- i) Disease agents suspected, ~~and~~ tests requested and purpose of the sampling.
- ii) Species, breed, sex, age and identity of the animals sampled, and trackability number when available.
- iii) Date ~~samples~~ specimens were collected and submitted.
- iv) List and type of ~~samples~~ specimens submitted with transport media used.
- v) Case history:
 - a) a) The clinical signs and their duration, including the temperature of sick animals, condition of mouth, eyes and feet, and milk or egg production data as relevant. Potentially pictures of the animals can be attached.
 - b) b) A list and description of the animals examined and the findings of the ante- and post-mortem examinations.
 - c) c) The length of time sick animals have been on the premises; if they are recent arrivals, from where did they originate.
 - d) d) The date of the first cases and of subsequent cases or losses, with, for tracking, any appropriate previous submission reference numbers.

vi) Ecological information

When sampling ectoparasites, trap locations and collection method, host association and environmental conditions of trapping are important associated metadata.

3. Epidemiological information

- i) A description of the spread of infection in the herd or flock.
- ii) The number of animals on the premise by species, the number of animals dead, the number showing clinical signs, and their age, sex and breed.
- iii) The type and standard of husbandry, including biosecurity measures and other relevant factors potentially associated with the occurrence of cases.
- iv) History of foreign travel by owner or of introduction of animals from other countries or regions.
- v) Any medication given to the animals, and when given.

-
- vi) Vaccination history describing the type of vaccines used and dates of application.
 - vii) Other observations about the disease, husbandry practices and other disease conditions present as well as ecological observations.

Shipping from the field to the laboratory requires careful planning to preserve specimen integrity and ensure biosafety. Specimens should be placed in appropriate containers following chapter 1.1.3 and international transport regulations (e.g. IATA). The cold chain should be maintained as appropriate for the specimen type and duration of transport. The choice of preservative, such as ethanol, proprietary nucleic acid preserving solution or formalin, should be compatible with the intended analysis. Accompanying documentation must be complete. Where appropriate sending samples should be compliant with international regulations, including CITES. Clear communication with the receiving laboratory prior to shipment is advisable to facilitate appropriate handling upon arrival and avoid delays that could compromise specimen quality.

D. RECEIPT, STORAGE AND ARCHIVES OF LABORATORY SUBMISSIONS

1. Reception of ~~samples~~ specimens

Receiving, unpacking and aliquoting specimens must be done in a way ~~to avoid cross-contamination in order that~~ avoids cross-contamination to guarantee reliable testing of samples and prevent exposure of personnel.

A risk assessment (RA) as outlined in chapter 1.1.4 should be performed before systems for handling biological agents and toxins are established ~~in order~~ to define the appropriate biosafety and laboratory biosecurity measures. The RA should lead to the development of stated policy and procedures for the operation of the whole process of receiving submissions to the laboratory.

Submissions delivered to the laboratory should be received in accordance with specified standard operating procedures by staff who are appropriately trained, and when possible are made aware of potential arrivals so that parcels are treated correctly upon reception. To enable appropriate specimen tracking the following information should be logged: a) the time of arrival, b) the sender, c) the person receiving the ~~samples~~ specimens, and d) the shipper with the tracking number. Where a specified chain of custody is required for the purposes of legal action or investigation the consignment should remain unopened and secured in a cool, dry place away from direct sunlight until authorised laboratory personnel are notified and available to receive the package and continue chain of custody. Laboratories should have a written operating procedure detailing the requirements to meet national legal requirements for such submissions.

1.1. Specimen reception area

Specimen reception areas should be equipped to facilitate the safe handling and processing of diagnostic submissions to avoid contamination of the work area, the personnel, cross-contamination among specimens and to allow easy disinfection in situations where specimen containers may have leaked. The specimen reception room should be clean with adequate bench space for organising submissions and paperwork. Depending on the number of submissions expected and depending on the RA the specimen reception area may be either a dedicated part of the diagnostic laboratory or a separate space outside the diagnostic laboratory.

The receiving room should contain an area dedicated to the unpacking of the specimens, with easily cleanable surfaces and trays and/or a biosafety cabinet, depending on the RA. There should be adequate and appropriate space to store specimens, either refrigerators or freezers, taking into consideration the time the specimens are to be stored. Specimen registration equipment such as computers, printers or logbooks should be available. A bar code system can be used to identify and track the specimens.

1.2. Submission unpacking, registration and preparation for further processing

Consignments should remain unopened until transferred to the specimen reception area for further processing. The submission should be unpacked and opened according to defined standard operation procedures. Surface decontamination should be considered to avoid cross-contamination and be part of the designated procedures arising from the RA.

Information to be recorded at specimen log-in includes the delivery source, the date the submission was consigned, the condition of the outer package, and the condition of inner packages (noting the presence of leaks or breakage), the condition of the specimen material, the inner package temperature, and any specific requests from the sender.

Further activities may include labelling of specimen containers, transfer of specimens to the laboratory and storage of specimens.

Packaging material should be disposed of appropriately according to national regulations which may include autoclave destruction of all packaging materials, depending on the RA.

Personal protective equipment (PPE) should be provided to protect the personnel. Minimal PPE is a laboratory coat and gloves. Depending on the relevant RA respiratory protection or effective splash protection (e.g. protective glasses) may be required as well.

After it has been determined that the submission contains the appropriate paperwork matching the specimens and that the specimens are in good condition appropriately trained laboratory personnel become responsible for transfer to the appropriate laboratory area, including maintenance of the chain of custody of the specimens as required. Only properly contained registered (identified) specimens should be transferred into the diagnostic laboratory. It is good practice, and at times a requirement for biosafety and laboratory biosecurity dependent on the RA, to enclose the submitted specimen containers in a secondary container to transfer the specimens safely within the laboratory.

1.3. Emergencies

A comprehensive RA will identify credible and foreseeable emergency scenarios, and be the basis for preparing a response plan. In particular, leaky ~~samples-specimens~~ represent a biohazard for the laboratory personnel and could contaminate the environment and other ~~samples-specimens~~. Written instructions on how to deal with broken or leaky tubes should be available in the sample reception area. Personnel should be trained and regular emergency exercises and simulations should take place.

~~Samples-Specimens~~ that are degraded or in a condition unacceptable for testing should be decontaminated and appropriately disposed of according to the laboratory's response plan as noted in the prior paragraph. Contaminated laboratory surfaces should be decontaminated with the appropriate disinfectant. ~~Sample-Specimen~~ rejection and discrepancies between the ~~samples-specimens~~ and accompanying paperwork should be resolved by contacting the sender immediately to resend a duplicate sample or to clarify paperwork.

2. ~~Sample-Specimen quality~~, storage and archives

Collections of well characterised specimens including infectious agents, infected tissues and fluids, as well as negative control tissues and fluids, are critical for future research and development efforts, for retrospective studies, epidemiological investigations, and for providing critical reference materials used in assay standardisation, validation, and proficiency testing programmes. In addition, specimens being investigated for legal purposes should be banked.

Materials routinely needed as reference standards and for assay validation are described in Chapter 1.1.5 *Quality management in veterinary testing laboratories*. The materials maintained in laboratory archives should be representative of the agents handled and the types of ~~samples-specimens~~ used in the different testing methods performed, which would variously include fresh and fixed tissues and fluids, paraffin-embedded tissues, and stabilised or otherwise preserved cultures. The World Federation for Culture Collections (WFCC: www.wfcc.info/collections) is a useful source of information and reference documentation for developing, maintaining, and sharing culture collections, and has published a comprehensive guide for establishing and operating microbial culture collections (WFCC, 2010). It is part of the remit of WOA Reference Laboratories to supply reference materials.

The principal components of any laboratory archive include the appropriate means of stabilisation and storage, a complete system of documentation and inventory of the material stored, and implementation of biosafety and laboratory biosecurity measures needed to manage the collection.

2.1. Specimen quality

Volume, quantity and integrity of specimens as well as types of containers, visible contamination, storage history and proper labelling are the first steps of the quality check of any specimen type. Other quality criteria are sample-type dependent. Bacterial contamination and red blood cell debris in serum samples can produce false-positive reactions in agglutination-type assays. Serological assays can be negatively impacted by haemolysis in the serum sample. Microbial contamination and haemolysis are significant concerns, especially when obtaining blood and serum samples from post-mortem animals.

2.1.2.2. Stabilisation and storage

The method of preserving tissues, fluids, and cultures will depend on their anticipated use(s). ~~Samples~~ Specimens and samples stored for periodic access such as assay reference materials should be aliquoted to avoid potential problems associated with repeated retrieval and return to storage. They may be stored separately from specimens or samples stored for historical, long-term preservation. Preservation of some aliquots for long-term nucleic acid preservation is desirable. Storage conditions should be managed to maintain viability, biochemical, and immunological properties ~~of the samples to~~ the maximum extent possible. Considerations for preserving the integrity of the specimens and samples must include protection from desiccation (e.g. as can happen in certain freezers), frequent or extreme temperature fluctuations, UV degradation, humidity, contamination, and the potential for loss of identification and associated archive documentation. Unique or valuable isolates and materials should be stabilised and stored using at least two different procedures and storage locations.

Storage at ultra-low temperatures (e.g. liquid nitrogen, cryopreservation in freezers at -140°C or lower) is considered the optimum method for long-term storage of biological materials. Storage at low-freezer temperatures of -80°C and -20°C is common for periods that may range from months to 5–10 years. Ultra-low freezing may not be a practical choice as it is expensive to maintain, but cost must be balanced with the fact that biological degradation of the ~~sample-specimens and samples~~ over time is an increasing risk at warmer freezer temperatures. Reference materials that are to be accessed with any regularity should be stored in appropriately-sized aliquots to allow access while minimising the number of times the “master stock” is handled. Repeated freezing and thawing ~~of samples~~ should be avoided as it can denature antigens, result in loss of viability of fastidious agents, and can precipitate the over-growth of contaminants or unwanted microorganisms in the sample.

Methods for stabilisation and storage of specimens and samples at room temperature range from commercially available technologies that largely target nucleic acid stabilisation, lyophilisation processes, to the relatively low-tech versions of drying fluids on filter paper disks or storage of biological ~~samples~~ specimens and samples in the presence of desiccating agents such as silica gel or grains of rice to absorb moisture.

Considerations such as speed at which a sample is frozen or chemically preserved, size and density of the material to be preserved, storage container and media, and ~~also~~ protocols for reconstitution, thawing, and reviving agents will vary with different tissues and agents. Whether the plan is to store samples frozen or at ambient temperature, for most tissues and groups of infectious agents there are specific preservatives, stabilisation protocols, and storage conditions that are considered optimal; the current published literature should be consulted.

2.2.2.3. Documentation and Inventory

Agents and tissues maintained in an archive must be correctly identified and sufficient supporting data that characterises the ~~sample-specimen~~ or agent must be recorded preferably in a machine-readable format (e.g. CSV [comma-separated values], JSON [java script object notation], XML, TXT [if structured properly], barcodes/QR codes, binary code). For reference materials, further documentation that authenticates the agent or tissue is required. The unique identity of the tissue, fluid, or agent and the storage location are best maintained in an electronic, machine readable format or paper inventory record which also documents the date the material was obtained, date and method of preservation, volume of material stored, source of the material including associated species, geographical location, and the clinical history of the donor animal and the disease situation of the flock or herd. Additional information is extremely useful and generally includes the original method of isolation/recovery, characterisation (e.g. available data on biochemical properties, antibody or antigen titre, and genetic sequence) as well as additional history on handling of the material (e.g. number of passages for infectious agents and cell lines, dates the archived material was frozen-thawed or rehydrated and the dates it was transferred to different storage conditions or containers). Inventories are most often organised by assigning a unique identifying number or alphanumeric code to each sample (sample container) that is cross-referenced to a database or inventory log. Inventory records can be manual data logs, computerised spreadsheets, or specialised computer programs. However the records are managed, they must be kept current and the information entered must be version controlled, stored with a backup in case of local system failure and traceable to its source. The identification of the individual making an entry or modification to the sample inventory should be recorded.

2.3.2.4. Biosafety and laboratory biosecurity

As a first step in establishing an archive, a laboratory biorisk assessment addressing biosafety and laboratory biosecurity issues, including any control or mitigation measures to be implemented, must be completed. As further defined in chapter 1.1.4, an appropriate risk assessment for archived samples

should address the technical competency needed of staff handling the tissues, fluids, and agents, with particular emphasis on those materials that are potentially infectious or toxic to workers, animals, and the environment in and around the laboratory. The laboratory should consider all biorisk management measures needed to protect the integrity of the sample, as well as the health of the workers and environment, from the time the original sample is received through the long-term storage and ultimate use or destruction of the material(s).

The appropriate level of laboratory biosecurity, including controlled access to the archived samples and inventory records, is an important consideration for laboratories maintaining biological inventories and archives. The laboratory should also have a back-up plan for the transfer or destruction of potentially dangerous archived materials in the event of power failures or other compromises to the storage environment. National and international regulations and legislation, including requirements for permits and licenses to receive, maintain, work with, and distribute specific agents and tissues must be followed for all laboratory archives. Current regulatory information ~~in regards to on~~ receiving and storage of biological materials can be found on the European Biological Resource Centre Network website (www.ebrcn.net/), in the WFCC guidelines (2010) and from relevant national government agencies.

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NB: FIRST ADOPTED IN 2008 AS COLLECTION AND SHIPMENT OF DIAGNOSTIC SPECIMENS.
MOST RECENT UPDATES ADOPTED IN 2013.

APPENDIX 1.1.2.1.

EPIDEMIOLOGICAL APPROACHES TO SAMPLING: SAMPLE SIZE CALCULATIONS

The type and number of ~~samples-specimens~~ needed depends on the desired purpose. In the case of wildlife, investigations require a case-by-case situation analysis depending on available data and the biology of the investigated species. Sample size calculation for each of the main purposes of testing, where random sampling has been used, may be approached as follows (see also WOAH Terrestrial Code):

1. Demonstrate freedom from infection in a defined population (country/zone/compartment/herd where the prevalence is apparently zero)

Frequently, the objective of sampling is to determine if a disease is present or absent in a population at a specific threshold (design prevalence). These sampling methods are needed to perform the scientifically based surveys specified in the WOAH *Terrestrial Animal Health Code* in order to determine freedom with and without vaccination as well as to re-establish freedom after outbreaks.

It is possible to calculate how many animals should be sampled from a herd/flock of a certain size, to achieve a 95% probability of detecting infection or previous exposure assumed to be present in a certain percentage of the animals. The following formula can be applied:

$$n \cong \frac{(1 - (1 - CL)^{1/D})(N - 1/2)(SeD - 1))}{Se}$$

Where

n : is the required sample size
 CL : is the confidence level (generally 0.95)
 N : is the population size
 D : is the number of diseased animals expected in the population
 Se : is the diagnostic sensitivity of the test used

For example, to determine the sample size required to detect with 95% confidence at least one infected animal in a herd of 500 animals at a design prevalence of 10%, the formula above would be used as follows (assuming perfect diagnostic sensitivity):

$$n \cong (1 - (1 - 0.95)^{1/50})(500 - 1/2)(50 - 1)) \cong 28$$

If the laboratory results are negative for all samples the epidemiologist can conclude, with 95% confidence, that the prevalence is lower than 10%. If the disease in question is highly infectious and it is unlikely that only 10% of the animals would be infected, the herd could be considered free. If, however, one or more samples are positive the epidemiologist may conclude, with 95% confidence, that the disease prevalence is at least 10%.

2. Certify freedom from infection or presence of the agent in individual animals or products for trade/movement/translocation purposes

The *Terrestrial Code* provides specific recommendations for trade purposes. Some are based on demonstration of disease freedom at a herd or flock level and others on testing of individual animals for export. When certification of a disease free herd or flock is recommended, the approach described in point 1 above can be followed to calculate the number of samples required.

In the case of testing individual animals, it is generally expected that all animals will be tested. The critical question in this case is related to the negative predictive value (NPV) of the test and the probability of having at least one false negative individual in a group. The negative predictive value of a test is defined as the probability that an animal is not infected given that it tested negative. The NPV is a function of the diagnostic sensitivity and specificity

of the test(s) used and the prevalence of the infection in the population where the animals come from. In general, the probability of having at least one false negative in a group is calculated as:

$$P(x \geq 1) = 1 - (1 - NPV)^n$$

The negative predictive value is calculated as follows:

$$NPV = \frac{TN}{TN + FN} = \frac{(1 - p)Sp}{(1 - p)Sp + p(1 - Se)}$$

Where

TN: is the true negative
FN: is the false negative
Se: is the diagnostic sensitivity
Sp: is the diagnostic specificity
p: is the prevalence
n: is the number of animals in the group

Additional information on quantifying these types of probabilities can be found in the WOA *Handbook on Import Risk Analysis for Animals and Animal Products, Quantitative Risk Analysis*.

3. Eradication of disease or elimination of infection from defined populations

The objective of surveillance in the event of an outbreak is to try to find any remaining pockets of infection. Sampling should be directed at populations having higher risk of exposure and where the agent is most likely to be found, such as animals exhibiting clinical signs or suspected to have been in contact with infected animals. If animals within a selected herd or flock are not exhibiting clinical signs, a representative sample, based on the formula presented under point 1 above for presence or absence of disease, should be collected.

4. Confirmatory diagnosis of suspect or clinical cases (includes confirmation of positive screening test)

Suspect or clinical cases with a positive screening test result should be retested with a confirmatory test. Usually, a test with high diagnostic sensitivity is used for screening purposes and one with high diagnostic specificity for confirmation. If the status of the herd or flock is of interest, animals exhibiting clinical signs compatible with the disease of interest should be sampled. This will increase the probability of confirming the infection. If required, the formula for presence or absence in point 1 above can be used to calculate the number of samples required. Given that sample collection is directed to animals exhibiting clinical signs, the design prevalence used can be relatively high, yielding a lower sample size.

5. Estimate prevalence of infection

Disease control programmes may need to periodically assess the impact of control measures. One of the key indicators of success is a reduction in the prevalence of the disease. To determine the prevalence of disease within a group of animals, the following formula can be used to determine the number of samples required.

$$n = \frac{Z^2 pq}{L^2}$$

Where

n: is the required sample size
Z: is the value of the Z distribution for the desired confidence level (usually 95%)
p: is the expected prevalence in the population
q: is 1-p
L: is the level of precision (or acceptable error)

There are no fixed rules to determine the level of precision (sometimes called also margin of error), the choice of it is left to the epidemiologist conducting the survey. However, finer levels of precision require larger sample sizes. The corresponding value of the Z distribution for 95% confidence is 1.96. To determine the prevalence of a disease with 95% confidence in a herd of 500 animals with an expected disease prevalence of 20%, at a level of precision of ±3%, the required sample size can be calculated:

$$n = \frac{1.96^2 \times 0.2 \times 0.8}{0.03^2} = \frac{0.6146}{0.0009} \cong 683$$

Note that in this case the required sample size is larger than the population, so the sample size will need to be adjusted to take account the population size (N):

$$n_{adj} = \frac{1}{1/n + 1/N} = \frac{1}{1/683 + 1/500} \cong 289$$

Therefore, 289 animals would have to be randomly sampled.

6. Determine immune status of individual animals or populations (post-vaccination).

Disease control programmes often rely on vaccination as a tool, in such cases it is important to assess immunity coverage and not merely count the number of animals or herds that have been vaccinated. The proportion of animals that need to be immunised to stop the spread of disease in a population is a function of the number of secondary infections arising from a single infected case (the reproductive number, R_0). For many infectious diseases the proportion needing immunity in order to control, disease spread is around 80%. Two approaches can be applied. If the objective is finding the proportion of immune animals, the formula for determining prevalence in point 5 above, can be applied. If, however, programme managers want to assess if herds have an immunity level at or above a certain threshold, the formula for presence or absence, in point 1 above, should be used. The difference in sample size varies greatly depending on the objective.

If the immune status of a herd of 500 animals that have all been vaccinated wants to be estimated, the following approaches can be followed.

6.1. Estimating the proportion of immune animals in a group

- ☐ Expected prevalence (of immune animals) 80%
- ☐ Precision, for this example assume 3%
- ☐ Confidence level 95%

$$n = \frac{1.96^2 \times 0.8 \times 0.2}{0.03^2} = \frac{0.6146}{0.0009} \cong 683$$

$$n_{adj} = \frac{1}{1/n + 1/N} = \frac{1}{1/683 + 1/500} \cong 289$$

6.2. Estimating immunity at a defined threshold

- ☐ Expected prevalence (of immune animals) 80%, i.e. 400 out of 500 animals
- ☐ Confidence level 95%
- ☐ Perfect diagnostic sensitivity

$$n \cong (1 - (1 - 0.95)^{1/400})(500 - 1/2(400 - 1)) \cong 3$$

If at least one of the three samples is positive to the test, the interpretation is, with 95% confidence, that the proportion of immune animals in the herd is at least 80%. If none of the samples test positive, the herd cannot be considered adequately immunised. Such an approach can be used to determine geographical locations or types of production systems where immune coverage is low and might need to be re-vaccinated.

On-line resources

Open Epi: <http://www.openepi.com/OE2.3/Menu/OpenEpiMenu.htm>

Free Calc: [Epitools - Sample size calculations http://epitools.ausvet.com.au/content.php?page=FreeCalc2](http://epitools.ausvet.com.au/content.php?page=FreeCalc2)

Win Episcopes: <http://www.winepi.net/>

[3_4-Field-sampling_Wiratsudakul.pdf](#)

Field Techniques for Population Sampling and Estimation – Monitoring Animal Populations and their Habitats: A Practitioner's Guide

Wildlife disease outbreak modelling open-source software: <https://scti.tools/outbreak/>. Provides a stochastic simulation of disease dynamics. A crucial tool in helping to predict the survival and potential for disease dynamics. <https://species360.org/species-conservation-toolkit-initiative-scti/>

APPENDIX 1.1.2.2.

SPECIMEN STORAGE METHODS AND THEIR DIAGNOSTIC SUITABILITY

<u>Storage method</u>	<u>Suitable specimen types</u>	<u>Permitted diagnostic tests</u>	<u>Notes/Limitations</u>
<u>Frozen (–20°C to –80°C)</u>	<u>Blood, plasma, serum, swabs, tissues, faeces, urine, arthropod specimens</u>	<u>PCR/real-time PCR (DNA/RNA), ELISA, virus isolation, biochemistry, serology, sequencing</u>	<u>Best for long-term nucleic acid and protein preservation. Avoid repeated freeze-thaw</u>
<u>Formalin-fixed (10% buffered)</u>	<u>Tissues, biopsies</u>	<u>Histopathology, immunohistochemistry (IHC)</u>	<u>Not suitable for PCR or DNA/RNA testing due to degradation and crosslinking</u>
<u>RNA preservative solution</u>	<u>Fresh tissues, swabs, faeces, blood</u>	<u>RNA-based assays (RT-PCR), transcriptomics</u>	<u>Preserves RNA well but not suitable for culture or histopathology</u>
<u>Ethanol (70–95%)</u>	<u>Faeces, tissues, swabs, parasites</u>	<u>PCR/qPCR, DNA sequencing (esp. barcoding, metagenomics)</u>	<u>Inhibits microbial culture. Ethanol concentration critical for DNA preservation</u>
<u>DNA stabilisation buffer (e.g. DESS, Longmire's)</u>	<u>Blood, swabs, tissue, saliva</u>	<u>DNA PCR, sequencing</u>	<u>Suitable for remote or room temperature field storage; not suitable for RNA</u>
<u>Refrigerated (4°C)</u>	<u>Urine, milk, swabs, CSF, blood</u>	<u>Culture, microscopy, antigen tests, limited PCR if processed quickly</u>	<u>Short-term only (typically ≤48 hours); degradation risk increases after that</u>
<u>Dry (e.g. filter paper, air-dried swabs)</u>	<u>Blood spots, faeces, swabs, hair, feathers</u>	<u>PCR (DNA), antibody elution, hormone analysis</u>	<u>Convenient in fieldwork. Humidity control (e.g. silica gel) improves long-term results</u>
<u>Cryopreserved (liquid nitrogen or –150°C)</u>	<u>Cells, viable viruses</u>	<u>Virus isolation, cell-based assays</u>	<u>Gold standard for preserving viable organisms or live cells. Requires specialised tools</u>
<u>Saline or sterile PBS (refrigerated)</u>	<u>Skin scrapings, mucus, tissues</u>	<u>Microscopy, culture, some PCR</u>	<u>Used short-term for microscopy or transport to labs</u>
<u>Transport media (VTM/UTM/MTM)</u>	<u>Swabs (oral, nasal, cloacal)</u>	<u>Viral PCR, antigen testing, culture (some media)</u>	<u>Required for many respiratory viruses (e.g. SARS-CoV-2, influenza)</u>

General recommendations: avoid mixing preservatives unless compatibility is confirmed.

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* *

MEETING OF THE BIOLOGICAL STANDARDS COMMISSION

Paris, 8–12 September 2025

CHAPTER 1.1.3.

TRANSPORT OF BIOLOGICAL MATERIALS

INTRODUCTION

The transport of biological materials, including infectious substances, is covered by international, regional or national regulations that are updated on a regular basis and are widely accessible via the internet, or through commercial and regulatory transportation affiliates. The transport of biological materials within a country and between countries will be explained in this chapter.

The international regulations for the transport of infectious substances by any mode of transport are based upon the Recommendations on the Transport of Dangerous Goods made by the Subcommittee of Experts on the Transport of Dangerous Goods (UN SCETDG), a subcommittee of the United Nations Economic and Social Council. The Recommendations are presented in the form of Model Regulations covering air, rail, road, sea and also include international mail. The World Health Organization (WHO) guidance document on “Transport of Infectious Substances” summarising the different transport regulations is regularly updated. Countries, other international organisations, international treaties and conventions such as the International Air Transport Association (IATA), the World Customs Organization (WCO), the Convention on International Trade in Endangered Species (CITES), and the Convention on Biodiversity (CBD), especially the Nagoya Protocol, provide additional guidance and regulations that should be considered in planning the transportation of biological materials.

In the interest of animal and human health, biological materials collected from animals must be transported safely, efficiently and legally from the place where they are collected to the place where they are analysed, studied or used. The collection of ~~specimens~~ samples from animals is covered in Chapter 1.1.2 Collection, submission and storage of diagnostic specimens.

For the purpose of this chapter, animals are defined as all members of the Kingdom Animalia except humans, and biological materials include specimens or samples from animals, cell cultures, zoonotic and animal microorganisms and genetically modified or synthetic organisms, and biological products such as vaccines and reagents.

All reasonable precautions have been taken to verify the information contained in this chapter, which provides guidance on proper transport of biological materials. However, this text does not replace local or global regulations. In no event is WOAHL liable for violations that arise from its use. Persons should always consult current local and global regulations and the logistics provider at the time of transport.

A. RESPONSIBILITIES

All personnel involved in the packaging, labelling and shipping of biological materials must be appropriately trained, certified, competent and knowledgeable of the relevant national, regional and international regulations.

Biological materials should be transported to ensure a rapid and reliable system for delivery to the recipient using individuals such as professional logistics service providers that are trained and competent in the shipping and transportation process.

The efficient transport and transfer of biological materials requires co-ordination between the sender (shipper, consignor), the logistic providers, the carrier and the recipient (consignee) to ensure safe transport and arrival on time and in proper condition.

The sender (shipper, consignor) is responsible for providing the applicable documentation (e.g. certifications, permits) required by the national authorities of the countries of export, transshipment and import as well as ensuring that the shipment also complies with all other applicable regulations, such as:

- i) Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization to the Convention on Biological Diversity (CBD): Biological material containing genetic resources as defined under the CBD may be subject to Access and Benefit-Sharing legislation in both the country where it is sourced and the country where it is sent.
- ii) CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora): All import, export, re-export and introduction from the sea of species covered by the Convention has to be authorised through a licensing system. Resolution Conf. 12.3. (Rev.CoP17) on Permits and Certificates, contains a section XII, regarding the use of simplified procedures to issue permits and certificates (https://cites-tsp.org/sites/default/files/resources_files/2022-12/E-Res-12-03-R17-min.pdf).

Procedures for incidents such as spills or theft of materials during transportation and any other realistic and foreseeable emergencies should be part of a risk management system in order to respond adequately to emergencies (for basic principles see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

1. The sender (shipper, consignor)

- i) Before any shipment of biological materials, the sender must be able to:
 - a) Identify and classify, pack (including temperature control), ensure quantity limits, mark and label the package of biological materials,
 - b) Ensure the correct documentation of all biological materials intended for transport,
 - c) Complete and produce a Shipper's Declaration for Dangerous Goods (DGD), when required,
 - d) Ensure biological materials are not forbidden for transport;
 - e) Ensure that the arrival date of the shipment does not fall on a week-end or day off;
 - f) In the event that ~~samples~~ biological materials need to be sent under controlled temperature conditions, measures to maintain a cold chain need to be carefully considered throughout the entire shipment (including accommodating any possible delays that may occur).
 - g) Ensure that the transportation of biological materials comply with quality and safety requirements. If there are special requirements for transportation, the transportation conditions should be confirmed.
- ii) Prepares necessary documentation, including permits, dispatch and shipping documents if necessary;
- iii) Notifies the recipient of transportation arrangements once these have been made, well in advance of the expected arrival time;
- iv) The air way bill (AWB) is the standard shipping document for shipping goods by air. While it is common practice for the air carrier or freight forwarder to complete the air waybill, the sender may be required to provide it;
- v) Makes advance arrangements with the recipient including investigating the need for import/export permits;
- vi) Makes advance arrangements with the carrier to ensure:
 - a) that the shipment will be accepted for appropriate transport;
 - b) that the shipment is undertaken by the most direct routing, as appropriate.
 - c) that a transport security plan/programme (TSP) is in place.

2. The carrier/courier

- i) The following measures must be taken by the carrier:
 - a) Routing: appropriate routing must be ensured, such as by the shortest or most secure route.
 - b) Transshipment: when transfers are necessary, precautions must be taken to assure special care, expeditious handling and monitoring of the substances in transit for ~~both~~ safety, cold chain maintenance (if required) and security purposes.
- ii) For air transport, the carrier is required by the regulations to use, when applicable, an acceptance checklist to verify that the shipment complies with:
 - a) marking and labelling requirements; and
 - b) documentation requirements.
- iii) Provide advice to the sender and assistance regarding the necessary shipping documents and instructions for their completion as well as correct packaging
- iv) Assists the sender in arranging the most appropriate routing and then confirms the routing and provides, if possible, ways to track the shipment;
- v) Maintains and archives documentation for shipment and transport.

3. The recipient (consignee)

- i) Obtains the necessary authorisation(s) from national authorities for the importation of the material;
- ii) Provides the sender with the required import permit(s), letter(s) of authorisation, or other document(s) required by the national authorities;
- iii) Arranges for the most timely and efficient collection on arrival;
- iv) Shall acknowledge receipt to the sender.

Shipments should not be dispatched until all the necessary arrangements between the sender, carrier and recipient have been made.

The transportation chain involves many more stakeholders with specific roles and responsibilities. These are explained in more details in the framework of aviation security in a joint ICAO (International Civil Aviation Organization) and WCO (World Customs Organization) brochure that can be accessed using the following link: <http://www.wcoomd.org/en/topics/facilitation/instrument-and-tools/tools/~media/4B167884A3064E78BCF5D29E29F4E57E.ashx>.

In addition, Material Transfer Agreements (MTA) should be considered because they:

- i) Protect the interests of all involved parties in relation to:
 - a) Intellectual property
 - b) Potential alternative uses
 - c) Commercial aspects
 - d) Liability to third parties
 - e) Potential further transfers/uses
- ii) Help to avoid misunderstandings around the use of materials
- iii) Clarify ownership of property

With reference to Article 4 of the “Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization (ABS) to the Convention on Biological Diversity”, the MTA is intended to establish a platform for the open exchange of materials among laboratories and to constitute Prior Informed Consent (PIC) on Mutually Agreeable Terms, while avoiding as much as possible the potential adverse impacts of the Nagoya requirements.

Appendix 3 is provided as a generic template for use in transfer of materials. It is intended to be modified and adapted as required to meet individual circumstances.

Appendix 3 does not constitute legal advice and users are responsible to ensure it meets their objectives and fulfils requirements of local legislation. Users are encouraged to consult their own legal professionals for advice on use of this template.

B. CLASSIFICATION AND CATEGORISATION

When transporting biological materials, the certified/ trained sender must determine whether the material should be classified as dangerous goods or not. Dangerous goods (hazardous materials, HAZMAT) are materials that can harm humans, animals and other living organisms, property, or the environment, and their transport is regulated by United Nations (UN) regulations¹. Dangerous goods are assigned a *UN number* and *proper shipping name* based on the classification of the dangerous goods. The transport regulations assign a packing instruction against the UN number and proper shipping name, to specify the packaging/packing method to ensure that the dangerous goods do not pose a hazard in transport. Of the biological materials that are discussed in this Chapter, infectious substances are classified as dangerous goods and are assigned to UN 2814, UN 2900, UN 3373, or UN 3291, as appropriate. In addition, Genetically Modified Microorganisms (GMMOs) and Genetically Modified Organisms (GMOs) are classified as Class 9 and assigned to UN 3245 if they are not classified as Category A or Category B.

Table 1. Summary of classification, categorisation, identification and packaging of infectious substances

Dangerous goods classifications	Categorisation	Proper shipping name ²	UN number ²	Packing instruction/ packaging requirements
Class 6, Division 6.2	Category A	Infectious substance, affecting humans	UN 2814	P620
		Infectious substance, affecting animals	UN 2900	
Class 6, Division 6.2	Category B	Biological substance, Category B	UN 3373	P650
Class 6, Division 6.2	Exempt human/animal specimens	Exempt human/animal specimens	N/A	Triple packaging to include a leakproof primary container. A leakproof secondary container with absorbent material and outer rigid packaging
not subject to dangerous goods regulations	Biological materials not subject to dangerous goods regulations	N/A	N/A	N/A
Class 9	GMMOs and GMOs that are not classified as Category A or B infectious substances	Genetically modified microorganisms; Genetically modified organisms	UN 3245	P904 (ICAO/IATA PI 959), IBC99

If it is likely that microorganisms that are present in the biological materials can cause harm to humans or animals then they must be assigned either to Category A or B.

The proper shipping name (see Table 1) must be supplemented with the technical name (scientific name of the pathogen) in parenthesis on the transport document, but not on the outer packaging. When the identity of the infectious substances to be transported are unknown, but are suspected of meeting the criteria for inclusion in category A, the words “suspected category A infectious substance” must be shown, in parenthesis, following the proper shipping name on the transport document.

1 http://www.unece.org/trans/danger/publi/unrec/rev13/13nature_e.html;

http://www.unece.org/trans/danger/publi/unrec/rev20/20files_e.html

<http://www.unece.org/>; <https://unece.org/transport/dangerous-goods/un-model-regulations-rev-23>

2 Dangerous goods are assigned UN numbers and proper shipping names according to their hazard classification and condition under the Dangerous Goods Regulations. See the Dangerous Goods List at pages 191–304 of the UN Model Regulations http://www.unece.org/fileadmin/DAM/trans/danger/publi/unrec/rev20/Rev20e_Vol1.pdf

1. Category A

A Category A substance is an infectious substance which is transported in a form that, when exposure to it occurs, is capable of causing permanent disability, life-threatening or fatal disease in otherwise healthy humans or animals. Assignment to UN 2814 or UN 2900 (see Table 1) must be based on the known medical history of the animal(s), signs and individual circumstances of the ~~specimen-sample~~ source, and endemic local disease conditions, or professional judgement concerning individual circumstances of the source, human or animal.

Some organisms are considered Category A only when in culture form (e.g. *Bacillus anthracis*, foot and mouth disease virus). Indicative examples of substances that meet these criteria are given in the Table 3. The table is not exhaustive. Infectious substances, including new or emerging pathogens, that do not appear in the Table but that meet the same criteria must be assigned to Category A. In addition, if there is doubt as to whether or not a substance meets the criteria it must be assigned to Category A.

Some infectious substances may have a high economic or trade impact on specific countries should there be release to the environment. Therefore, other infectious substances may be added to the list by individual countries (e.g. cultures of Newcastle disease virus where the virus is exotic to the country or region).

Medical or clinical waste containing Category A infectious substances shall be assigned to UN 2814 or UN 2900 as appropriate. **Solid** medical waste containing Category A infectious substances generated from the medical treatment of humans or veterinary treatment of animals may be assigned to UN 3549. It should be noted that Medical or clinical waste from bioresearch or liquid waste must not be assigned to UN3549.

2. Category B

Biological materials containing pathogens that do not meet the criteria for Category A (i.e. do not cause life-threatening disease to humans or animals) shall be assigned to Category B (UN 3373).

Typically, a specimen with a high likelihood to contain pathogenic organisms shipped for disease diagnosis (e.g. confirmatory diagnosis of suspected or clinical cases, specimens for differential diagnosis, such as blood samples for classical swine fever or sheep pox diagnostics or throat samples from chickens for avian influenza) can be assigned to Category B.

It is important to note that unlike cultures, *patient specimens* that may contain infectious microorganisms listed as 'cultures only' in Table 3 (Category A infectious substances) do not require Category A transport practices. For these specimens Category B transport practice should be applied. In this case, although directly collected specimens (e.g. serum) can be shipped as Category B, pure cultures of the same pathogens must follow the requirements of Category A due to the characteristics of the specific organism. Some examples are classical swine fever virus isolates or sheep pox virus isolates (see Table 3). Specimens from animals intentionally infected with Category A pathogens must be sent as Category A, even if they are assigned to Category A (cultures only).

Shipments of cultures of non-category A agents can be assigned to Category B.

Medical or clinical waste containing Category B infectious substances shall be assigned to UN 3291.

3. Exempt ~~specimens-samples~~

Animal ~~specimens-samples~~ for which there is minimal likelihood that pathogens are present can be transported as Exempt ~~Specimens-Samples~~. Examples of specimens in the veterinary field that may be transported as exempt include specimens from surveillance studies, export controls of healthy animals (e.g. certification of freedom from classical swine fever) or determination of immune status of individual animals or populations (post-vaccination).

These specimens are not subject to dangerous goods regulations if the specimen is transported in a packaging that will prevent any leakage and that is marked appropriately (triple packaging principle, see Section C. *Packaging* and Figure 6 of Appendix 1.1.3.2).

4. Biological materials not subject to Dangerous Goods Regulations

Based on the known medical history of the animal(s), signs and individual circumstances of the source of the biological materials, and endemic local disease conditions, the following *are not subject to dangerous goods regulations, unless they meet the criteria for inclusion in another class (such as Class 9)*:

- i) biological materials known to not contain infectious substances
- ii) biological materials containing microorganisms that are non-pathogenic to humans or animals;
- iii) biological materials in a form in which any pathogens present have been neutralised or inactivated such that they no longer pose a health risk;
- iv) Environmental specimens (including food and water specimens) that are not considered to pose a significant risk of infection;
- v) Dried blood spots, collected by applying a drop of blood onto absorbent material.

Note: Materials not subject to dangerous goods regulations may need to be risk assessed before proceeding with their shipment. There may be specific regulations in place in some countries for the shipment, export or import of nucleic acids.

5. Contaminated items

These listed below are also included in infectious substances in the international regulations on transport of dangerous goods, however the details are not discussed in this chapter. For more information see UN Model Regulations, paragraphs 2.6.3.2.3.3 and .9 respectively.

Table 2. Summary of classification, categorisation, identification and packaging of contaminated items with infectious substances

Dangerous goods classifications	Categorisation	Proper shipping name ³	UN number ³	Package
Class 6, Division 6.2	Category A	Medical* devices or equipment contaminated with or containing infectious substances in Category A	UN2814, UN2900 as appropriate	Must be marked "Used Medical Device" or "Used Medical Equipment"
Class 6, Division 6.2	Exemption when condition is met	Medical* devices, medical equipment	N/A	See UN Model Regulations 2.6.3.2.3.9 and IATA Dangerous Goods Regulations (DGR) 3.6.2.2.3.9
Class 6, Division 6.2	Category A	Medical* waste, Category A, affecting humans, solid ; Medical waste, Category A, affecting animals only, solid	UN 3549	P622, LP622
Class 6, Division 6.2	Category B	Clinical waste, Unspecified, n.o.s. (not otherwise specified); (Bio) medical waste, n.o.s.; Regulated medical waste, n.o.s.	UN3291	P621 (PI622), IBC620, LP621

*including veterinary use

6. Infectious substances included in Category A

Table 3. Indicative examples of infectious substances included in Category A

UN number and proper shipping name	Microorganism
UN 2814 Infectious substance, affecting humans	<i>Bacillus anthracis</i> (cultures only)
	<i>Brucella abortus</i> (cultures only)
	<i>Brucella melitensis</i> (cultures only)

3 Dangerous goods are assigned UN numbers and proper shipping names according to their hazard classification and condition under the Dangerous Goods Regulations. See the Dangerous Goods List at pages 191–304 of the UN Model Regulations http://www.unece.org/fileadmin/DAM/trans/danger/publi/unrec/rev20/Rev20e_Vol1.pdf

UN number and proper shipping name	Microorganism
	<i>Brucella suis</i> (cultures only)
	<i>Burkholderia mallei</i> – <i>Pseudomonas mallei</i> – Glanders (cultures only)
	<i>Burkholderia pseudomallei</i> – <i>Pseudomonas pseudomallei</i> (cultures only)
	<i>Chlamydia psittaci</i> – avian strains (cultures only)
	<i>Clostridium botulinum</i> (cultures only)
	<i>Coccidioides immitis</i> (cultures only)
	<i>Coxiella burnetii</i> (cultures only)
	Crimean–Congo haemorrhagic fever virus
	Dengue virus (cultures only)
	Eastern equine encephalomyelitis virus (cultures only)
	<i>Escherichia coli</i> , verotoxigenic (cultures only) ⁴
	Ebola virus
	Flexal virus
	<i>Francisella tularensis</i> (cultures only)
	Guanarito virus
	Hantaan virus
	Hantaviruses causing haemorrhagic fever with renal syndrome
	Hendra virus
	Hepatitis B virus (cultures only)
	Herpes B virus (cultures only)
	Human immunodeficiency virus (cultures only)
	Highly pathogenic avian influenza virus (cultures only)
	Japanese encephalitis virus (cultures only)
	Junin virus
	Kyasanur Forest disease virus
	Lassa virus
	Machupo virus
	Marburg virus
	Monkeypox virus
	<i>Mycobacterium tuberculosis</i> (cultures only) ¹
	Nipah virus
	Omsk haemorrhagic fever virus
	Poliovirus (cultures only)
	Rabies virus (cultures only)
	<i>Rickettsia prowazekii</i> (cultures only)
	<i>Rickettsia rickettsii</i> (cultures only)
	Rift Valley fever virus (cultures only)
	Russian spring–summer encephalitis virus (cultures only)

⁴ For surface transport (ADR) nevertheless, when the cultures are intended for diagnostic or clinical purposes, they may be classified as infectious substances of Category B.

UN number and proper shipping name	Microorganism
	Sabia virus
	<i>Shigella dysenteriae</i> type 1 (cultures only)
	Tick-borne encephalitis virus (cultures only)
	Variola virus
	Venezuelan equine encephalitis virus (cultures only)
	West Nile virus (cultures only)
	Yellow fever virus (cultures only)
	<i>Yersinia pestis</i> (cultures only)
UN 2900 Infectious substance, affecting animals only	African swine fever virus (cultures only)
	Avian paramyxovirus Type 1 – Velogenic Newcastle disease virus (cultures only)
	Classical swine fever virus (cultures only)
	Foot and mouth disease virus (cultures only)
	Goatpox virus (cultures only)
	Lumpy skin disease virus (cultures only)
	<i>Mycoplasma mycoides</i> – contagious bovine pleuropneumonia (cultures only)
	Peste des petits ruminants virus (cultures only)
	Rinderpest virus (cultures only ⁵)
	Sheep-pox virus (cultures only)
	Swine vesicular disease virus (cultures only)
	Vesicular stomatitis virus (cultures only)

C. PACKAGING

1. Principles

All biological materials should be packaged and transported in accordance with local, national and international regulations. The procedures should minimise the risk of exposure for those engaged in transportation and should protect the environment and susceptible animal populations from potential exposures. Additionally, ineffective packaging that does not protect specimens or preservatives (e.g. ice) from damage or prevent leakage will likely delay the delivery of the shipment to the laboratory, delaying or preventing critical laboratory analyses from being performed. Biological materials should always be packaged and transported to protect the integrity of the specimens, as well as to avoid cross-contaminating other specimens and environmental contamination. Minimum requirements for the transport of ~~specimens~~ biological materials follow the principle of triple packaging, consisting of three layers as described below:

- i) a primary leak-proof receptacle;
- ii) a secondary leak-proof packaging with absorbent material;
- iii) a rigid outer packaging.

1.1. Primary receptacle

A primary receptacle, leak-proof for liquids or sift-proof for solids containing the specimen. Primary receptacle(s) must be packed into the secondary packaging with enough absorbent material (e.g. cellulose wadding, paper towels, household paper, cotton balls) to absorb all fluid in case of breakage. Even though the regulations do not prohibit glass, primary receptacles should preferably be non-

⁵ Subject to prior approval by the FAO-WOAH rinderpest secretariat

breakable. In addition, they must not contain any sharps (e.g. vacutainer with needle), particularly when using soft secondary or outer containers. If screw cap vials are used, they shall be secured by e.g. tape. A flip-top vial must not be used.

1.2. Secondary packaging

A second durable, leak-proof packaging to enclose and protect the primary receptacle(s) (e.g. sealed plastic bag, plastic container, screwcap can).

For transport by air, the primary receptacle or the secondary packaging shall be capable of withstanding, without leakage, an internal pressure of 95 kPa (0.95 bar) in the range of -40°C to $+55^{\circ}\text{C}$ (-40°F to $+130^{\circ}\text{F}$).

1.3. Outer packaging

Secondary packaging is placed in a rigid outer shipping packaging (e.g. sturdy insulated fibre board box) with suitable cushioning material. Outer packaging protects the contents from outside influences, such as physical damage, while in transit.

2. Category A

Due to the highly hazardous nature of the Category A samples the packaging must meet special requirements. The principle of triple packaging applies here, and the transport containers and outer packaging must meet the criteria defined in the relevant regulations. Category A must only be transported in packaging that meets the United Nations class 6.2 specifications, complies with Packing Instruction P620 and have passed specific tests and with UN specification marking as P620. This ensures that strict performance criteria are met; tests for compliance with these criteria include a 9-metre (29.5 feet) drop test, a puncture test, a pressure test and a stacking test. The packages are labelled to provide information about the contents of the package, the nature of the hazard and the packaging standards applied.

Marking and labelling is as follows (see Figure 3 of Appendix 1.1.3.2):

- i) *The delivery address (consignee) and sender's details (shipper), as well as 24/7 emergency contact details including named persons with telephone numbers to guarantee safe delivery.*
- ii) *The proper shipping name and the UN number.*

<i>Proper shipping name</i>	<i>UN number</i>
<i>INFECTIOUS SUBSTANCE, AFFECTING HUMANS</i>	<i>UN2814</i>
<i>INFECTIOUS SUBSTANCE, AFFECTING ANIMALS only</i>	<i>UN2900</i>

- iii) *The Infectious Substance label (Figure 1).*

NB: This label is only for Category A. This label must not be used when shipping Category B.



Fig. 1. Infectious Substance label for the transport of Category A.

- iv) *UN specification marking for P620 packaging (printed on the box).*
- v) *Orientation label, Cargo only label, if required (depending on the Net Weight [kg] of the infectious substance in a P620 box).*

The exact details can be found in P620 Packing Instruction⁶.

For air transport:

- i) The primary receptacle or secondary packaging must be capable of withstanding, without leakage, an internal pressure of 95 kPa. The primary receptacle or secondary packaging must also be capable of withstanding temperatures in the range of -40°C to $+55^{\circ}\text{C}$;
- ii) For liquids: the net quantity of infectious substances per one P620 box shall not exceed 50 ml for transport in cargo space of a passenger aircraft; and must not contain more than 4 litres (contain multiple primary receptacles totalling more than 4 litres) for transport on a cargo only aircraft;
- iii) For solids: the net quantity of infectious substances per one P620 box shall not exceed 50 g for transport in cargo space of a passenger aircraft, and must not contain more than 4 kg (even if containing multiple primary receptacles totalling more than 4 kg) for transport on a cargo only aircraft. This quantity limit doesn't apply for animal parts, organs and whole carcasses.
- iv) The three triple packaging principle has to be adopted accordingly using appropriate packaging systems;
- v) The entire package must have been tested and complies with Packing Instruction P620.

For further information on marking and labelling of the Category A shipment package, see P620 Packing Instruction for UN Nos 2814 and 2900⁶

3. Category B

Category B must be transported in a packaging that complies with the requirements of packing instruction P650. The approval of the box by the government is not required, thus UN specification marking is not required.

Marking is as follows:

- i) Packages should be clearly labelled with the delivery address and sender's details to guarantee safe delivery in time at the correct destination.
- ii) Label with the *proper shipping name* in letters at least 6 mm high: "BIOLOGICAL SUBSTANCE, CATEGORY B" (Figure 2)
- iii) In addition to the proper shipping name, the mark shown below (UN3373 in diamond) is used for shipments of Category B substances. The UN3373 mark must always be visible on the outer packaging.

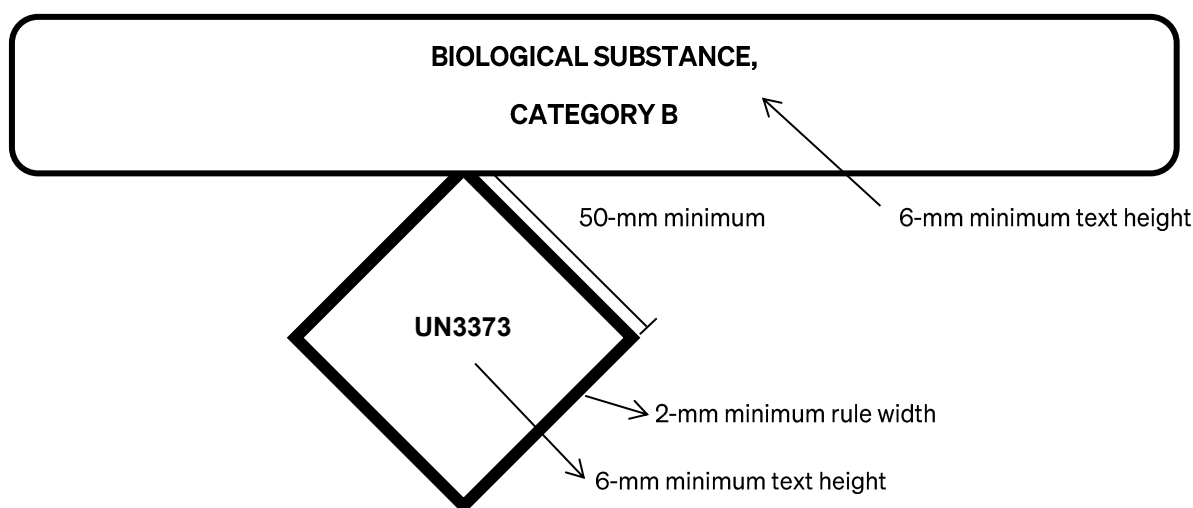


Fig. 2. UN3373 mark for the transport of Category B substances.

Additional requirements do apply as for category A for international shipment and air transport. One of the main differences between P650 and P620 is the reduced drop-test to 1.2 metres (4 feet).

For air transport:

- i) The primary receptacle or secondary packaging must be capable of withstanding, without leakage, an internal pressure of 95 kPa. The primary receptacle or secondary packaging must also be capable of withstanding temperatures in the range of –40°C to +55°C;
- ii) For liquids: no primary receptacle shall exceed 1 litre and the outer packaging must not contain more than 4 litres (contain multiple primary receptacles totalling more than 4 litres);
- iii) For solids: the outer packaging must not contain more than 4 kg. This restriction doesn't apply for animal parts, organs and whole carcasses.

The exact details can be found in P650 Packing Instruction for UN No. 3373⁷.

4. Exempt specimens

Biological materials for which there is a minimal likelihood that pathogens are present are not subject to regulation if the specimen is carried in a packaging which will prevent any leakage and which is marked with the words "Exempt animal specimens", as appropriate. The triple packaging system must still be applied.

5. Biological materials not subject to Dangerous Goods Regulations

This exemption refers to biological materials that do not contain infectious substances and are therefore not subject to dangerous goods regulations (such as class 6.2) and any packaging requirements, *unless they meet the criteria for inclusion in another class (such as class 9)*.

Note: There may be specific regulations in place in some countries for the shipment, export or import of nucleic acids.

6. Overpack

"Overpack" is the term used when one or more packages are combined to form one unit and sent to the same destination by a single shipper. When refrigerants are used to protect contents, the overpacks may comprise insulated vessels or flasks. Whenever an overpack is used, the required marks and labels shown on the outer packaging must be repeated on the outermost layer of the overpack, except for the UN specification marking on P620. This requirement applies to all infectious substances including Categories A and B. Overpacks are also required to be marked with the word "overpack".

Combining different categories of infectious substance in a same overpack is permissible however in this case outer labelling should indicate the highest category included in the package.

7. Cold chain transportation

7.1. Cold chain requirements

Most biological materials require a cold chain to maintain their integrity. Vaccines can be especially sensitive to temperature fluctuations and must always be transported within their safe temperature range to maintain potency and efficacy.

- i) Confirm the safe temperature range for transportation of the material. For transportation of vaccines, follow the manufacturer's instructions.
- ii) Package the material in accordance with its classification, including appropriate temperature monitoring equipment for transportation of vaccines.
- iii) Use the refrigerant that will maintain the correct temperature range during transportation.
- iv) Label the outer packaging with the recommended transportation temperature range to ensure correct handling during shipping and transfers.

7.2. Refrigerants

7 Page 82 at: http://www.unece.org/fileadmin/DAM/trans/danger/publi/unrec/rev20/Rev20e_Vol2.pdf

Refrigerants may be used to stabilise ~~specimens~~ biological materials during transport.

Ice, ice packs or dry ice shall be placed outside the secondary receptacle. Wet ice shall be placed in a leak-proof container; the outer packaging or overpack shall also be leak-proof.

Dry ice (solid carbon dioxide) must not be placed inside the primary or secondary receptacle because of the risk of explosion. A specially designed insulated packaging may be used to contain dry ice, typically a polystyrene or waxed-treated cardboard box to prevent leakage and maintain temperature. The packaging must permit the release of carbon dioxide gas if dry ice is used and the package (the outer packaging or the overpack) shall be marked “UN 1845” and “Carbon dioxide, solid as coolant” or “Dry ice as coolant” and the weight of the dry ice in Kilograms should also be indicated on the labelling. The package must also bear the Class 9 – Miscellaneous hazard label.

The secondary receptacle shall be secured within the outer package to maintain the original orientation of the inner packages after the refrigerant has melted or dissipated.

If liquid nitrogen is used as a refrigerant, additional requirements have to be followed according to the relevant regulations for dangerous goods (Division 2.2, UN 1977). Information on Dry Shipper is available in p19 of WHO Guidance on regulations for the transport of infectious substances 2023–2024⁸.

D. ADDITIONAL CONSIDERATIONS

In addition to the transport regulations described above, other international agreements might be applicable.

1. CITES

The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) is an international agreement between governments with the aim to ensure that international trade in specimens of wild animals and plants does not threaten their survival. The Convention is in effect in 183 Parties (including one economic integration organisation, the European Union).

Some specimens to be transported from one country to another may be derived from species covered by CITES (roughly 5,600 animal species and 30,000 plant species). Depending on the classification of the species in one of the three Appendices of the Convention and the movement involved, a CITES export permit, both an export and import permit, or re-export certificates may be required. The appropriate documents must be obtained from National CITES Management Authorities. Simplified procedures for the issuance of permits and certificates exist to facilitate and expedite trade in biological specimens from CITES-listed species.

There may be some variation from one country to another in their CITES trade requirements (some countries take stricter domestic measures and some countries have added additional species requirements for permits in addition to the CITES lists), therefore It is always advisable to check the national legislation that applies.

Further information on CITES: <https://cites.org>

2. Nagoya protocol and access and benefit-sharing

“Fair and equitable sharing of benefits arising from the utilisation of genetic resources” (Access and Benefit Sharing, or ABS) is one of the three objectives of the Convention on Biodiversity (CBD). The CBD confirms that States have sovereign rights over their genetic resources, including animals, plants, fungi and microorganisms. Consequently, States may choose to regulate access to these, requiring researchers both within and outside their borders to seek permission. Permission (*Prior Informed Consent* or PIC) will be the responsibility of the State and maybe other stakeholders, and the conditions for sharing benefits (*Mutually Agreed Terms* or MAT) may be agreed with a range of actors, including laboratories.

In 2014 the *Nagoya Protocol on Access and Benefit Sharing* (NP) was agreed by the Parties of the CBD. Nagoya Protocol requirements have been implemented in a large number of countries. This obliges the Parties to take compliance measures and monitor researchers and others utilising genetic resources (accessed from other Parties) within their jurisdiction. Biological material containing genetic resources as defined under the CBD may be subject to national Access and Benefit-Sharing (ABS) legislation in both the country where it is sourced and the country where it is sent. This may include compliance with the NP if both countries are Party to the NP. To discover whether this is the case, consult the ABS Clearing House (<https://absch.cbd.int/>), and ask the National Focal Point of both

8 <https://iris.who.int/bitstream/handle/10665/376214/9789240089525-eng.pdf?sequence=1>

countries (contact details on ABS Clearing House). If the source country has ABS legislation it may be necessary to seek a permit (Prior Informed Consent and Mutually Agreed Terms) prior to international transport of the material.

Benefits may be monetary or non-monetary. "Utilisation of genetic resources" is defined by the CBD as "means to conduct research and development of the genetic and/or biochemical composition of genetic resources, including through the application of biotechnology as defined in Article 2 of the Convention". Consequently, many activities carried out by WOA Members and their constituents may be classified as ABS.

Compliance will require researchers to produce documentary evidence that genetic resources were accessed with appropriate PIC and MAT, and declaring the type of utilisation being undertaken. This information will be transmitted to the provider country to check if the information accords with their records. There is no exemption for organisms of veterinary importance from ABS provisions in the NP, but countries where they are being accessed may choose to make this distinction and exemption.

Article 8 of the Nagoya Protocol states *"In the development and implementation of its access and benefit-sharing legislation or regulatory requirements, each Party shall: ... (b) Pay due regard to cases of present or imminent emergencies that threaten or damage human, animal or plant health, as determined nationally or internationally. Parties may take into consideration the need for expeditious access to genetic resources and expeditious fair and equitable sharing of benefits arising out of the use of such genetic resources, including access to affordable treatments by those in need, especially in developing countries;"*. However, this is not required and countries may choose to take no action. In addition, there is a 90-day grace period allowed to complete required documents after transport of the biological material.

If the research or diagnostic work on biological material of veterinary concern is considered 'utilisation' by the country (Party) in which it takes place the researcher will be required to provide information, including:

- i) The Internationally Recognised Certificate of Compliance (number) from the ABS Clearing House, if available; or
 - a) ABS Permit reference
 - b) Evidence of Prior Informed Consent
 - c) Evidence of Mutually Agreed Terms
 - d) The entity to whom PIC and MAT was granted
- ii) The Provider Country
- iii) Date and place of access
- iv) Description of the Genetic Resources

This information should be included in the documents with the transported biological material.

Although this documentation is only required if both providing country and recipient country are Party to the Nagoya Protocol the provider may have Access and Benefit-Sharing legislation even if not a Party. This should be respected and any documentation required, including permits, Prior Informed Consent and Mutually Agreed Terms, acquired and dispatched with the material.

Further information can be found on:

- i) The ABS Clearing House – <https://absch.cbd.int/>
- ii) The CBD Website – <https://www.cbd.int/abs/default.shtml>

E. REFERENCES AND FURTHER READING

WHO Guidance on regulations for the transport of infectious substances 2023–2024: applicable as of 1 October 2023 covering transport regulations on national and international and air transport by different means:
<https://iris.who.int/handle/10665/254788>

Swiss Expert Committee on Biosafety on Transport of biological substances; practical explanation on how to transport biological substances according to the specific dangerous goods transport regulations:

<https://www.efbs.admin.ch/index.php?id=462&L=3>

IATA: <http://www.iata.org/whatwedo/cargo/dgr/Documents/infectious-substance-classification-DGR56-en.pdf>

F. ADDITIONAL INFORMATION ON THE UNITED NATIONS SYSTEM FOR THE TRANSPORT OF DANGEROUS GOODS

The United Nations dangerous goods web site provides comprehensive detail concerning the United Nations Recommendations on the Transport of Dangerous Goods. It also provides links to the modal agencies:

<http://www.unece.org/trans/danger/danger.html>

The site below provides the full text of the United Nations Recommendations on the Transport of Dangerous Goods – Model Regulations, which can be downloaded in PDF format. Readers wishing to see the text relating to the transport of infectious substances should download Part 2, Part 4 and Part 5 of the Recommendations:

http://www.unece.org/trans/danger/publi/unrec/rev20/20files_e.html

The site below provides the full text of the European Agreement concerning the International Carriage of Dangerous Goods by Road (ADR) of 2017, which entered into force on 1 January 2017, which can be downloaded in PDF format. Readers wishing to study the text relating to the transport of infectious substances should download Part 2 (2.2.62), Part 4 (search P620, P650) and Part 5:

<http://www.unece.org/trans/danger/publi/adr/adr2017/17contentse0.html> and

http://www.unece.org/trans/danger/publi/adn/adn2017/17files_e0.html

Contracting parties to the various conventions for the transport of dangerous goods can be found on a number of web sites:

- Air** ICAO: <http://www.icao.int/Pages/default.aspx> and <https://www.icao.int/safety/DangerousGoods/Pages/StateVariationPage.aspx>
IATA: <http://www.iata.org/whatwedo/cargo/dgr/Documents/infectious-substance-classification-DGR56-en.pdf>
- Rail** RID (Intergovernmental Organisation for International Carriage by Rail): <http://www.otif.org/>. RID is primarily for the countries of Europe, North Africa and the Middle East. There are a number of countries (mainly Eastern Europe and Asia that apply RID through the Organization for Cooperation of Railways (OSJD); details of RID membership can be found at <http://www.otif.org/en>
- Road** ADR: <https://unece.org/about-adr> (lists competent authorities)
- Sea** IMO (International Maritime Organization): <http://www.imo.org>
- Post** UPU (Universal Postal Union): <http://www.upu.int/>

NB: FIRST ADOPTED IN 1992 AS SAMPLING METHODS.
MOST RECENT UPDATES ADOPTED IN 2025.

APPENDIX 1.1.3.1.

DEFINITIONS

The following definitions are for the purposes of this chapter only. For general definitions please see the Glossary.

- **Biological products**

Biological products are those products derived from living organisms which are manufactured and distributed in accordance with the requirements of appropriate national authorities, which may have special licensing requirements, and are used either for prevention, treatment, or diagnosis of disease in humans or animals, or for development, experimental or investigational purposes related thereto. They include, but are not limited to, finished or unfinished products such as vaccines.

- **Cultures**

Cultures are the result of a process by which pathogens are intentionally propagated. This definition does not include human or animal patient specimens as defined below.

- **Infectious substances**

For the purposes of transport, infectious substances are defined as substances which are known or are reasonably expected to contain pathogens. Pathogens are defined as microorganisms (including bacteria, viruses, parasites, fungi) and other agents such as prions, which can cause disease in humans or animals. Infectious substances are further classified according to risk into two categories.

- **Genetically modified microorganisms (GMMOs) and organisms (GMOs)**

Genetically modified microorganisms not meeting the definition of infectious substance are classified in Class 9 (Miscellaneous dangerous substances and articles, including environmentally hazardous substances). GMMOs and GMOs are not subject to dangerous goods regulations when authorised for use by the competent authorities of the countries of origin, transit and destination. Genetically modified live animals shall be transported under terms and conditions of the competent authorities of the countries of origin and destination. DNA, RNA or plasmids are not considered as GMMO and not subject to dangerous goods regulations.

- **Medical or clinical wastes**

Medical or clinical wastes are wastes derived from the veterinary treatment of animals, the medical treatment of humans or from bio-research.

- **Patient specimens**

Patient specimens are those, collected directly from humans or animals, including, but not limited to, excreta, secreta, blood and its components, tissue and tissue fluid swabs, and body parts being transported for purposes such as research, diagnosis, investigational activities, disease treatment and prevention.

*
* *

APPENDIX 1.1.3.2.

EXAMPLE OF THE TRIPLE PACKAGING SYSTEM (IATA RECOMMENDATIONS) FOR THE PACKING AND LABELLING OF DIFFERENT TYPES OF BIOLOGICAL MATERIALS

Fig. 3. Example of triple packaging system for the packaging and labelling of Category A, UN2814 and UN2900 infectious substances
(Figure kindly provided by IATA, Montreal, Canada).

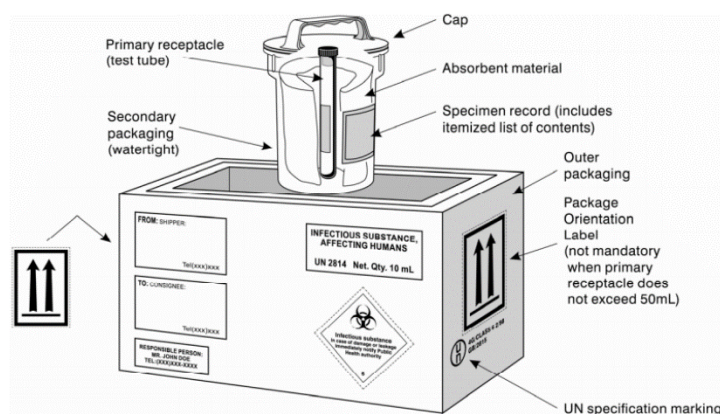


Fig. 4. Example of the triple packaging system for the packaging and labelling of Category B, UN3373 infectious substances (Figure kindly provided by IATA, Montreal, Canada).

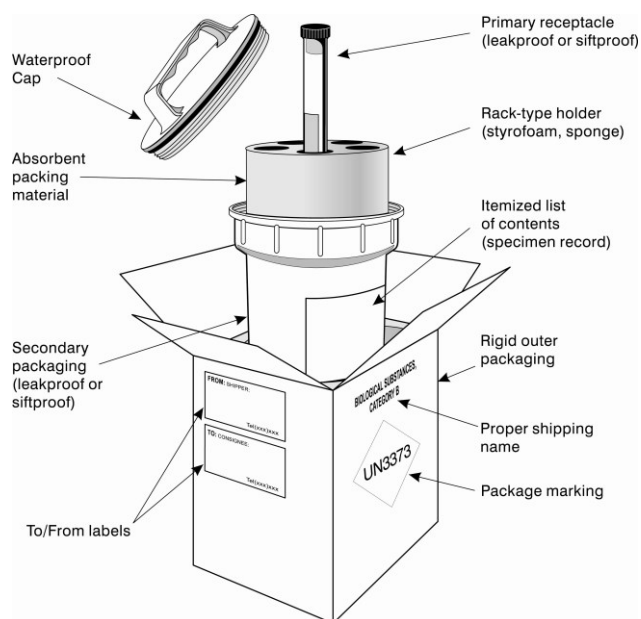


Fig. 5. Example of the triple packaging system for the packing and labelling of Category B, UN3373 infectious substances with non-rigid leakproof secondary packaging (Figure kindly provided by IATA, Montreal, Canada).

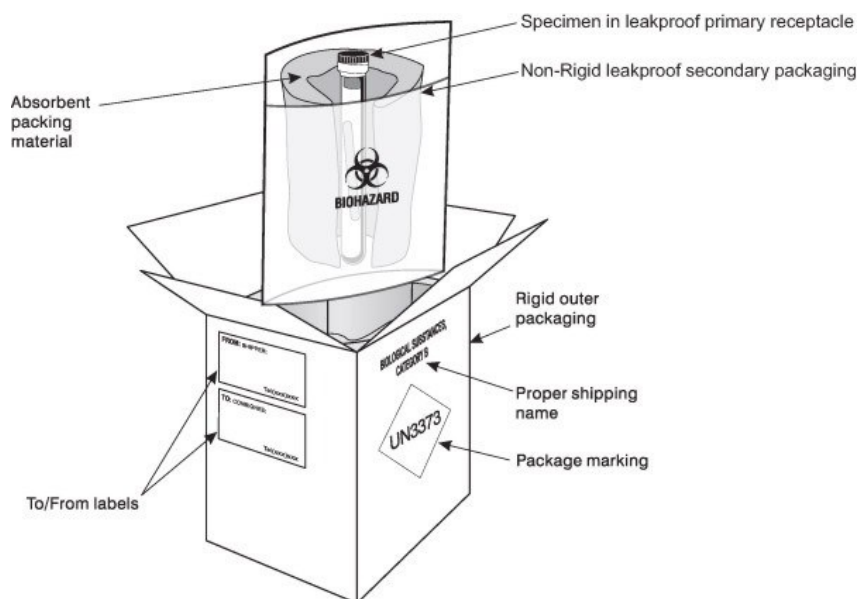
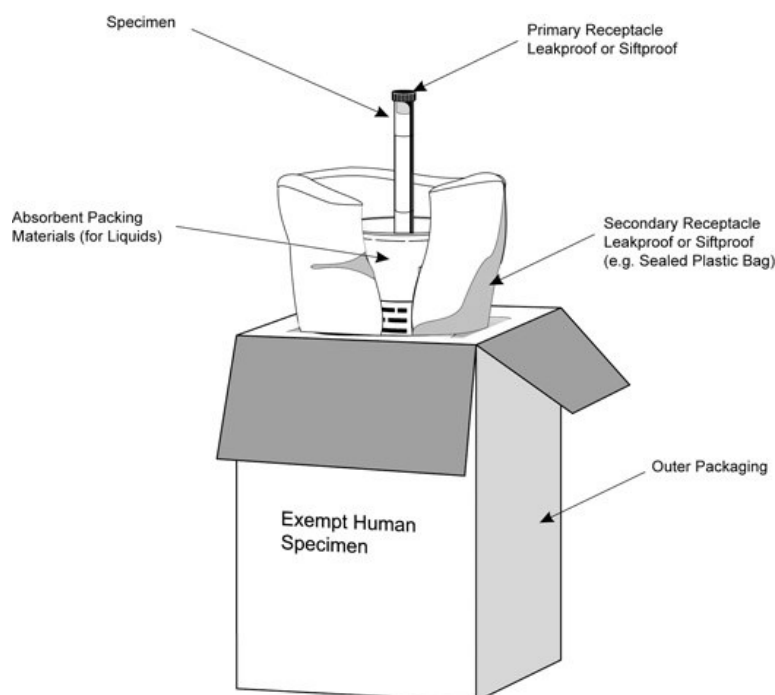


Fig. 6. Example of the triple packaging system for the packing and labelling of Exempt specimen (Figure kindly provided by IATA, Montreal, Canada).



APPENDIX 1.1.3.3.

MATERIAL TRANSFER AGREEMENT

MATERIAL TRANSFER AGREEMENT

BETWEEN

PROVIDER

Organisation:
Address:
Country:

PROVIDER SCIENTIST

Title and name:
Organisation:
Address:

AND

RECIPIENT

Organisation:
Address:
Country:

RECIPIENT SCIENTIST

Title and name:
Organisation:
Address:

ORIGINAL MATERIAL

Description of the material being transferred

SHIPPING ADDRESS

Title and name:
Address:

I. OBJECTIVE AND PURPOSE

[Insert a short statement about the objectives and intended purpose of the agreement and background to the parties to the agreement.]

II. DEFINITIONS

Provider

Organisation providing the *original material*. The name and address of this party will be specified in the first page of this MTA.

Provider scientist

The name and address of this party will be specified in an implementing letter.

Recipient

Organisation receiving the *original material*. The name and address of this party will be specified in an implementing letter.

Recipient scientist

The name and address of this party will be specified in an implementing letter.

Original material

The description of the material being transferred will be specified in an implementing letter.

Material

Original material, progeny, and unmodified derivatives. The *material* shall not include: (a) *modifications*, or (b) other substances created by the *recipient* through the use of the *material* which are not *modifications, progeny, or unmodified derivatives*.

Progeny

Unmodified descendant from the *material*, such as virus from virus, cell from cell, or organism from organism.

Unmodified derivatives

Substances created by the *recipient* which constitute an unmodified functional subunit or product expressed by the *original material*. Some examples include: subclones of unmodified cell lines, purified or fractionated subsets of the *original material*, proteins expressed by DNA/RNA supplied by the *provider*, or monoclonal antibodies secreted by a hybridoma cell line.

Modifications

Substances created by the *recipient* which contain/incorporate the *material*.

Commercial purposes

The sale, lease, license, or other transfer of the *material* or *modifications* to a for-profit organisation. *Commercial purposes* shall also include uses of the *material* or *modifications* by any organisation, including *recipient*, to perform contract research, to screen compound libraries, to produce or manufacture products for general sale, or to conduct research activities that result in any sale, lease, license, or transfer of the *material* or *modifications* to a for-profit organisation. However, industrially sponsored academic research shall not be considered a use of the *material* or *modifications* for *commercial purposes* per se, unless any of the above conditions of this definition are met.

Non-profit organisation(s)

A university or other institution of higher education or an organisation exempt from taxation or any nonprofit scientific or educational organisation qualified under a state nonprofit organisation statute. As used herein, the term also includes government agencies.

Agreement

Material Transfer Agreement (MTA)

III. TERMS AND CONDITIONS OF THIS AGREEMENT

Ownership

The *provider* retains ownership of the *material*, including any *material* contained or incorporated in *modifications*.

The *recipient* retains ownership of:

- (a) *modifications* (except that, the *provider* retains ownership rights to the *material* included therein);
- (b) those substances created through the use of the *material* or *modifications*, but which are not *progeny*, *unmodified derivatives* or *modifications* (i.e., do not contain the *original material*, *progeny*, *unmodified derivatives*). If either 2 (a) or 2 (b) results from the collaborative efforts of the *provider* and the *recipient*, joint ownership may be negotiated.

Non-commercial use

The *recipient* and the *recipient scientist* agree that the *material* is to be used solely for either teaching, non-commercial research or academic research purposes. In this Agreement, non-commercial research purpose and academic research purpose mean that the *material* cannot be used for *commercial purposes*, and the *recipient* may not exploit commercially the results, inventions, discoveries or know-how which incorporates the *materials* for its own benefit nor for a third party, without the consent of the *provider*.

Ownership of the results, inventions, discoveries or know-how generated by the *recipient* using the *material* shall rest with the *recipient*. Nevertheless, any results, inventions, discoveries or know-how which contain or incorporate the *material*, generated by the *recipient* using the *material* ("*modifications*") shall be jointly owned by the *provider* and the *recipient*. However, both the *provider* and the *recipient* agree that should the *recipient* having completed work under this MTA wish to use the *material* or *modifications* for commercial purposes it will be necessary for the *recipient* to negotiate the terms of a license Agreement with the *provider*. No right are given, implied or intended by this Agreement or the material transfer other than those explicitly stated in this Agreement.

Distribution to third parties

This *material* should be considered a property of the *provider*. The *recipient* therefore agrees to retain control over this material, and further agrees not to transfer the *material* to third parties or to personnel of the *recipient* not working under the supervision of the *recipient scientist*. The *recipient* agrees to refer to the *provider* any request for the *material* from anyone other than those persons working under the *recipient scientist's* direct supervision. The *provider* reserves the right to distribute the *material* to others and to use it for its own purposes.

The *recipient* shall have the right, without restrictions, to distribute substances created by the *recipient* through the use of the *original material* only if those substances are not *progeny*, *unmodified derivatives*, or *modifications*.

Under a separate agreement at least as protective of the *provider's* rights, the *recipient* may distribute *modifications* to *non-profit organisation(s)* for non-commercial research purposes and academic research purposes only, subject to prior written notice to the *provider*.

Confidentiality

The *recipient* agrees to treat the *materials* as it would treat its own confidential and proprietary information and at least no less than a reasonable degree of care, and to take all reasonable precautions to prevent unauthorised disclosure to any third party of the *material* which it receives hereunder. The *provider* agrees to keep confidential that the *recipient* is using the *material*.

Publications

This Agreement shall not be interpreted to prevent or delay publication of research findings resulting from the use of the *material* or the *modifications*. The *recipient scientist* agrees to provide appropriate acknowledgement of the source of the *material* in all publications.

Material use liability

The *material* is provided as a service to the research community at large. It is provided without warranty of merchantability or fitness for a particular purpose or any other warranty, express or implied. No indemnification for any damages is intended or provided under this Agreement. Each party should accept liability for their own actions. The parties make no express or implied warranty as to any matter whatsoever, including the conditions of the research or any invention or product, whether tangible or intangible, made, or developed under this Agreement, or the ownership, merchantability, or fitness for a particular purpose of the research or any invention or product. The parties further make no warranty that the use of any invention or other intellectual property or product contributed, made or developed under this Agreement will not infringe any patent or other intellectual property right. In no event, will any party be liable to any other party for compensatory, punitive, exemplary or consequential damages.

Misuse, dual use and biosafety

The *recipient* accepts full responsibility for the safety of the research and warrants that the research will be performed in accordance with all local or national laws, rules and regulations. In particular, this *material* will only be used for research purposes by the *recipient* in its laboratory under suitable containment conditions.

Under the terms of this Agreement, the *material* may not be used in human beings.

Termination of the Agreement

The term of this Agreement is 2 years as of the effective date of termination, unless an extension is mutually agreed by the *provider* and the *recipient*. At the end of this term, the Agreement shall automatically terminate. Upon the effective date of termination, or if requested, the deferred effective date of termination, the *recipient* will discontinue its use of the *material* and will, upon direction of the *provider*, return or destroy any remaining *material*. The cost of return or destruction will be taken by the *recipient*.

Dispute

Any dispute arising under this Agreement instituted against the *recipient* by the *provider* shall be brought in the court of the *recipient's* country of domicile. Any claims and proceedings against the *provider* by the *recipient* shall be brought in the courts of the *provider's* country of domicile.

Modification of the Agreement and signatures

This agreement may not be modified, in whole or in part, except by the written consent of both parties. If any provision of this Agreement may be signed in counterpart, and by the parties hereto or separate counterparts, each of which shall be deemed an original.

This Agreement is effective when signed by all parties. The parties executing it certify that their respective organisations have accepted the terms of this Agreement, and further agree to be bound by the terms, for the transfer specified above.

Recipient responsibility

The Recipient undertakes to use the Material in full compliance with any national and international applicable law, including any disposition and guidelines regarding health and scientific research. In particular, the Material having intrinsic health risk shall be handled in full respect of the specific law and in compliance with all the necessary precautions.

The Recipient represents that within its laboratories:

- Access to the Material, Progeny and Modification will be restricted to personnel capable and qualified to safely handle those substances, using appropriate containment;
- Recipient shall use the utmost precaution to minimise any risk of harm to persons and property and to safeguard them from theft or misuse.

The Recipient also acknowledges that in no event the Material applies directly or indirectly to humans.

The Recipient assumes all liability for any and all third party damages and claims arising out of or relating to this Agreement, including the receipt, use, handling, storage, conservation of the Material. To the extent permitted by applicable law, the Recipient agrees to indemnify, defend and hold harmless the provider against all third party claims, losses, expenses and damages, including reasonable attorney's fees.

The provider shall have no liability towards the Recipient or its employees in the event that the Material and/or Derivatives infringe any intellectual property rights of third parties. The provider makes no warranties for the absence of any third party industrial property rights on the Material.

IV. PAGE OF SIGNATURES

AUTHORISED SIGNATURE OF THE PROVIDER SCIENTIST

Signature:
Title and print name:
Date:

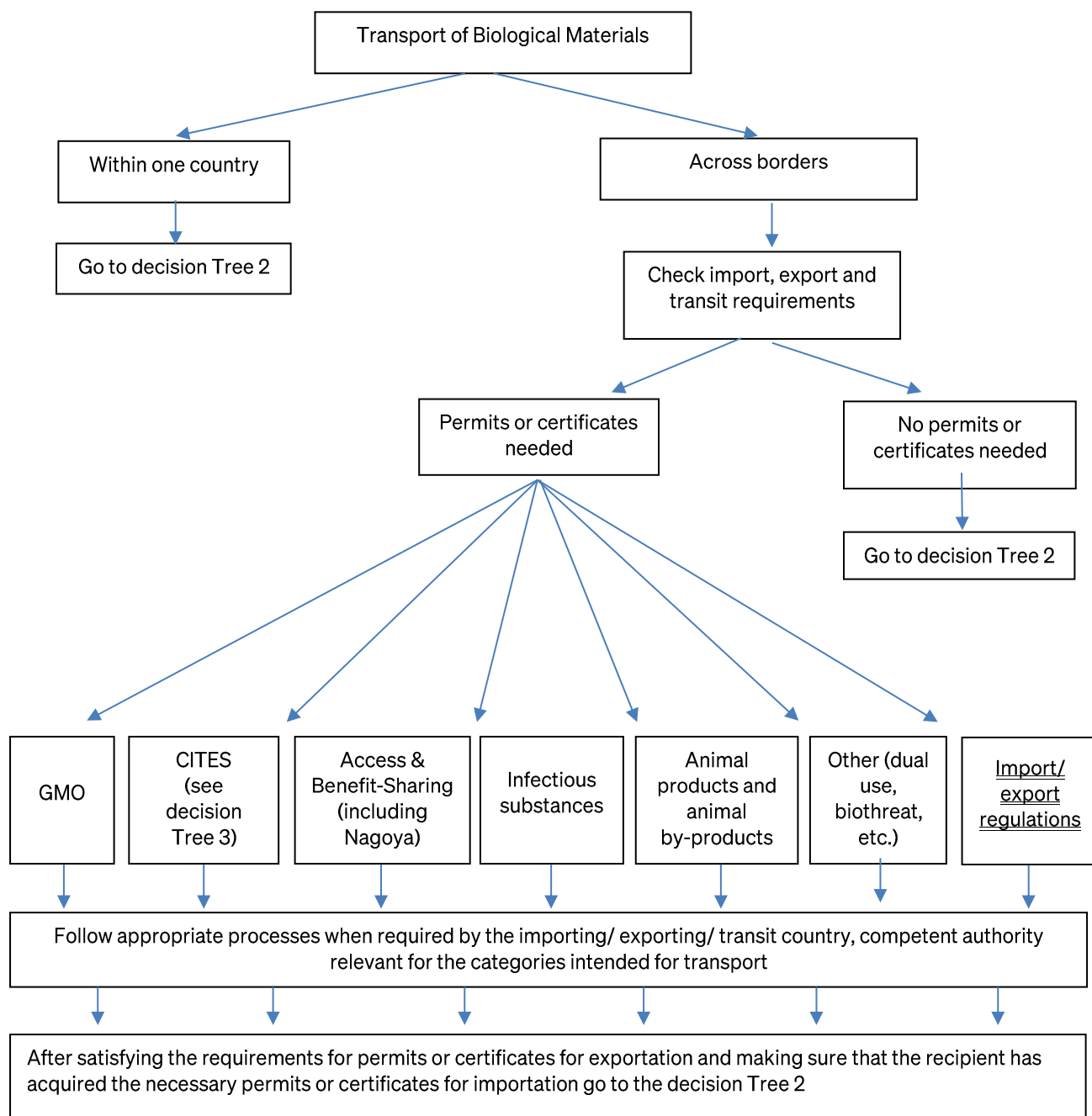
AUTHORISED SIGNATURE OF THE RECIPIENT SCIENTIST

Signature:
Title and print name:
Date:

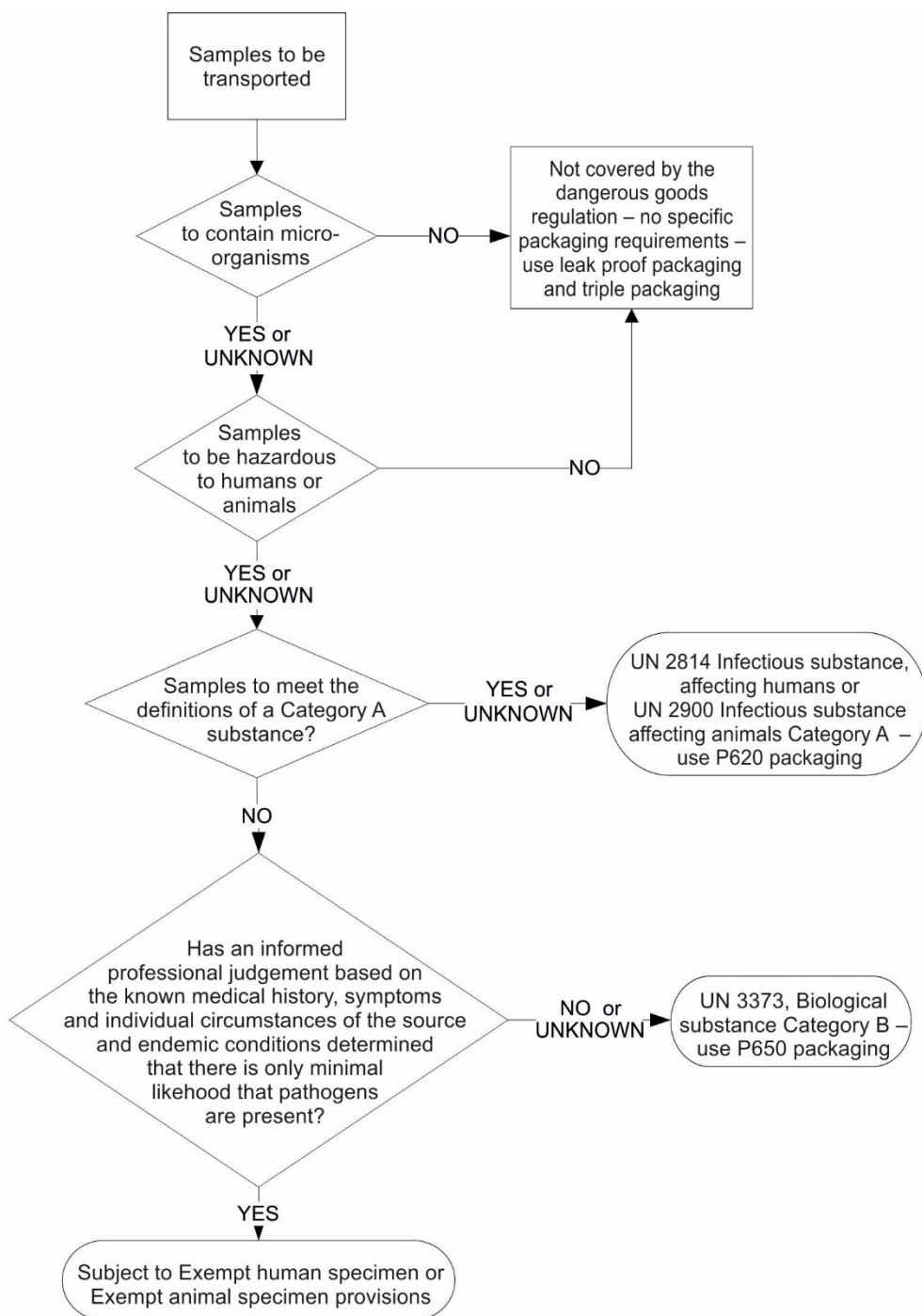
APPENDIX 1.1.3.4.

DECISION TREES FOR THE TRANSPORT REQUIREMENTS OF BIOLOGICAL MATERIALS

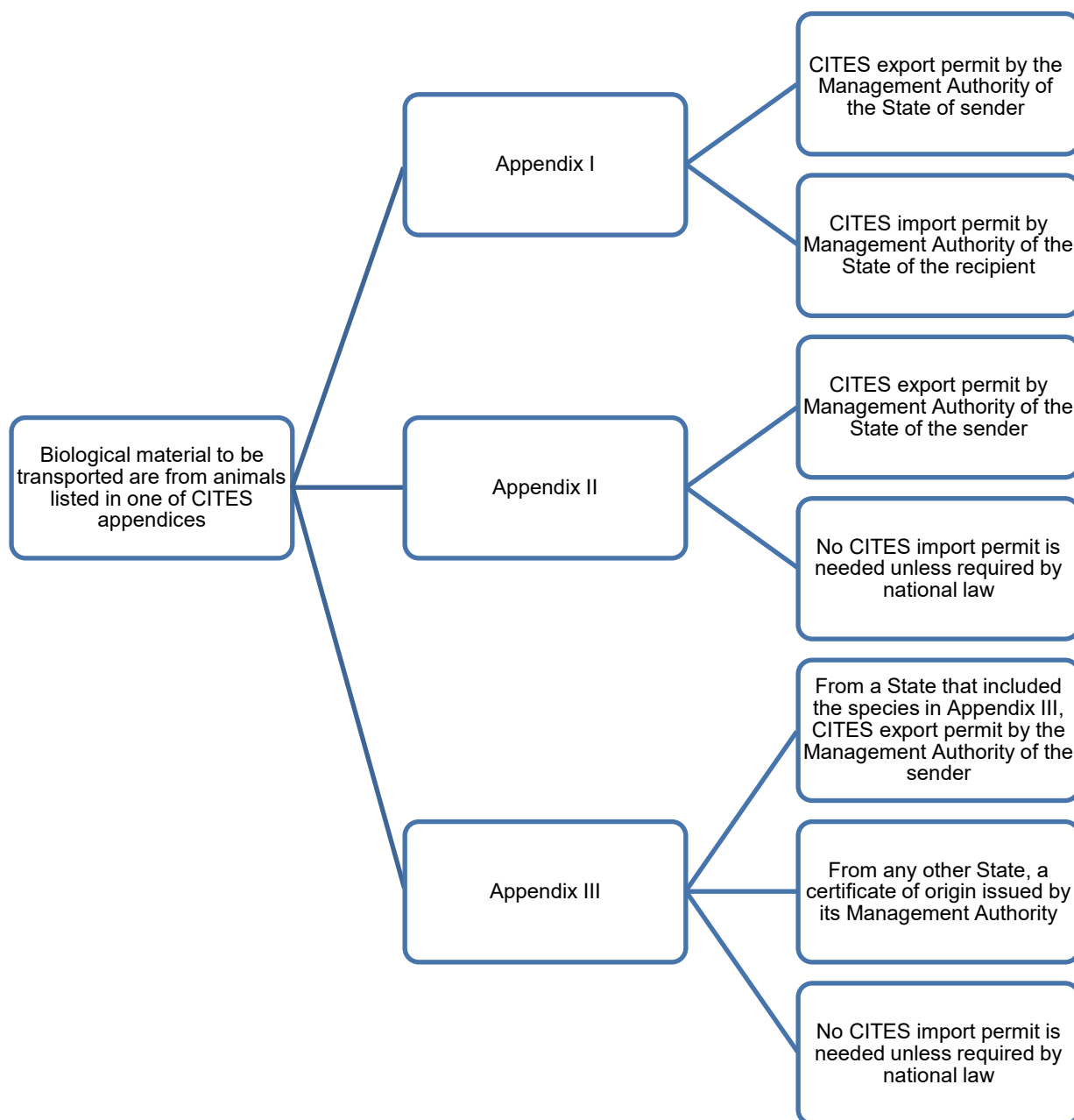
Decision Tree 1:



Decision Tree 2



Decision Tree 3



**Annex 6. Chapter 3.1.8. Foot and mouth disease (infection with foot and mouth disease virus)
(appendices only)**

MEETING OF THE BIOLOGICAL STANDARDS COMMISSION

Paris, 8–12 September 2025

Table 1. Test methods available for the diagnosis of FMD 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)						
Virus isolation	□□□	☑□□	☑☑☑	☑☑☑	□□□	□□□
Antigen detection ELISA	□□□	□□□	☑☑☑	☑☑☑	□□□	□□□
LFD	□□□	□□□	☑☑☑	☑☑☑	□□□	□□□
Real-time RT-PCR	☑□□	☑□□	☑☑☑	☑☑☑	☑□□	□□□
Conventional RT-PCR	☑□□	☑□□	☑☑☑	☑☑☑	☑□□	□□□
Detection of immune response						
NSP Ab ELISA	☑☑☑	☑☑□	☑☑☑	☑☑☑	☑☑☑	□□□
SP Ab ELISA ^(b)	☑☑□	☑☑□	☑☑☑	☑☑☑	☑☑□	☑☑☑
VNT ^(b)	☑☑□	☑☑□	☑☑☑	☑☑☑	☑☑□	☑☑☑

Key: ☑☑☑ = recommended for this purpose; ☑☑□ recommended but has limitations;

☑□□ = suitable in very limited circumstances; □□□ = not appropriate for this purpose.

ELISA = enzyme linked immunosorbent assay; LFD: lateral flow device; RT-PCR = reverse-transcriptase polymerase chain reaction;

NSP Ab ELISA = ELISA for antibodies against nonstructural proteins; SP Ab ELISA = ELISA for antibodies against structural proteins; VNT = Virus neutralisation test.

^(a)It is essential to confirm the presence of FMDV following virus isolation by an antigen or nucleic acid detection test.

^(b)The tests do not distinguish infected from vaccinated animals.

Appendix 1
Foot and mouth disease
Intended purpose of test: population freedom from infection

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
Real-time RT-PCR ☒ ☐ ☐	<u>[1] Testing often focuses on oral swabs and probang samples to identify sub-clinical cases and carrier ruminants (other sample types such as lymph nodes collected at abattoirs and bulk milk are also used for this purpose)</u>	<u>[1] High (approaching 100%), where there are animals that have been recently infected, but lower where infection has occurred historically</u>	<u>[1] Test purpose validated using samples from experimental studies</u>	<u>[1] See references and Appendix 4: Confirmation of clinical cases</u>	<u>[1] Real-time RT-PCR can be automated allowing large number of samples to be tested</u> <u>[2] Oral swabs and milk are simple to collect, time efficient, require less restraint and are relatively non-invasive</u>	<u>[1] Ability to recognise FMDV-infected animals is dependent on when samples are taken as well as technique used</u> <u>[2] Collection of probang samples requires specific training and is semi-invasive</u> <u>[3] More expensive than ELISA methods</u>	<u>Jamal <i>et al.</i> (2012)</u> <u>Stenfeldt <i>et al.</i> (2013)</u>
RT-PCR ☒ ☐ ☐	<u>[1] Testing often focuses on oral swabs and probang samples to identify sub-clinical cases and carrier ruminants</u>	<u>[1] Usually lower sensitivity compared with real-time RT-PCR</u>	<u>[1] See above descriptions for real-time RT-PCR as there is limited information on the use of RT-PCR for this purpose</u>	<u>[1] See reference and Appendix 4: Confirmation of clinical cases</u>	<u>[1] Oral swabs are simple to take, time efficient, require less restraint and are relatively non-invasive</u>	<u>[1] Screening large numbers of samples need to carefully consider the scope for cross-contamination (false positives)</u> <u>[2] Collection of probang samples requires specific training and is semi-invasive</u>	<u>Parida <i>et al.</i> (2005)</u>
NSP Ab ELISA ☒ ☒ ☒	<u>[1] Sera</u>	<u>[1] DSe between 95% and 100%</u> <u>[2] DSp between 98% and 100%</u> <u>[3] various NSP ELISA kits exist and these will have different DSe and DSp</u>	<u>[1] Samples from FMDV susceptible species collected as part of FMDV control programmes</u> <u>[2] This purpose is also supported by sera collected from experimentally vaccinated and/or infected animals</u>	<u>[1] See references</u>	<u>[1] High diagnostic sensitivity in non-vaccinated populations</u> <u>[2] A single test covers all FMDV serotypes</u> <u>[3] Tests are usually appropriate for all susceptible species</u> <u>[4] Easy to perform, suitable for large-scale application or automation</u> <u>[5] Simple-to-use commercial kits are available</u> <u>[6] Ideal for surveillance, control, and eradication</u>	<u>[1] This test is ideal to screen populations, but specificity is <100% therefore, confirmatory testing is often required</u> <u>[2] Lower test sensitivity should be expected if animals have been previously vaccinated</u>	<u>Bergmann <i>et al.</i> (2003)</u> <u>Brocchi <i>et al.</i> (2006)</u> <u>Browning <i>et al.</i> (2020)</u> <u>Bruderer <i>et al.</i> (2004)</u> <u>Campos <i>et al.</i> (2008)</u> <u>Lu <i>et al.</i> (2007)</u> <u>Malirat <i>et al.</i> (1998)</u> <u>Paton <i>et al.</i> (2006)</u> <u>Sharma <i>et al.</i> (2014)</u> <u>Sorensen <i>et al.</i> (2005)</u>
SP Ab ELISA ☒ ☒ ☐	<u>[1] Sera</u>	<u>[1] DSe between 93% and 100%</u>	<u>[1] Samples from FMDV susceptible species collected as part of FMDV control programmes</u>	<u>[1] See references and Appendix 3: Contribute to</u>	<u>[1] Simple-to-use commercial kits are available</u> <u>[2] SP-abs appear early after infection or vaccination so SP-</u>	<u>[1] Cannot differentiate between antibodies generated after vaccination or infection. Therefore, SP Ab ELISAs</u>	<u>Hamblin <i>et al.</i> (1986)</u> <u>Hamblin <i>et al.</i> (1987)</u> <u>Ludi <i>et al.</i> (2022)</u>

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
			<u>[2] This purpose is also supported by testing of sera collected from experimentally infected animals</u>	<u>eradication policies</u>	<u>ELISAs have higher diagnostic sensitivity compared with NSP ELISAs</u> <u>[3] Tests are usually appropriate for all susceptible species</u> <u>[4] Easy to perform, suitable for large-scale application or automation</u>	<u>cannot be used if vaccines are used</u> <u>[2] Tests have high sensitivity for FMDV-specific antibodies but often have poor inter-serotype specificity. Therefore, serotype confirmation using another method may be required</u>	<u>Mackay <i>et al.</i> (2001)</u> <u>Paiba <i>et al.</i> (2004)</u>
<u>VNT</u> ☒ ☒ ☐	<u>[1] Sera</u>	<u>[1] DSp≈100%</u> <u>[2] DSe≈100%</u> <u>[3] Test performance is influenced by the specific antigen used</u> <u>[4] Test conditions (cells and condition of cell culture) influence test performance</u>	<u>[1] Samples from FMDV susceptible species collected as part of FMDV control programmes</u> <u>[2] This purpose is also supported by sera collected from experimentally infected animals</u>	<u>[1] See references and Appendix 3: Contribute to eradication policies</u>	<u>[1] Test that is often used for confirmatory purposes with high sensitivity and specificity</u>	<u>[1] Test requires 1–3 days for execution</u> <u>[2] Maintenance and use of live virus and tissue culture cells, thus biocontainment restriction procedures are required</u>	<u>Willems <i>et al.</i> (2020)</u> <u>Hamblin <i>et al.</i> (1986)</u>

Appendix 2

Foot and mouth disease

Intended purpose of test: individual animal freedom from infection prior to movement

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Virus Isolation</u> ☒ ☐ ☐	<u>[1] Testing of samples from FMDV antibody-negative animals (blood and swabs) to rule out pre-clinical/sub-clinical infection</u> <u>[2] Testing of probang samples can be used to confirm FMDV-antibody results</u>	<u>[1] Considered to be the gold standard method</u> <u>[2] Test performance is dependent upon the cell line and culture conditions that are used</u> <u>[3] For appropriate samples collected from acutely infected animals, DSe and DSp can approach 100% using highly sensitive cell lines (example LFBKαVβ6)</u>	<u>[1] This test purpose is supported by data generated from experimental studies in cattle and other species</u>	<u>[1] See references and Appendix 4: Confirmation of clinical cases</u>	<u>[1] Potentially useful for subsequent studies for virus characterisation including vaccine selection</u>	<u>[1] Needs confirmatory testing to confirm the agent causing CPE is FMDV (e.g. Ag-ELISA or sequencing)</u> <u>[2] Material submitted for testing must be in good condition requiring that a cold chain is maintained from sample collection to the laboratory</u> <u>[3] Testing can be impacted by gross bacterial/fungal contamination, despite the use of antibiotics in the cell culture media</u> <u>[4] Time-consuming and specialist facilities are needed (with appropriate biocontainment)</u>	<u>Ferris <i>et al.</i> (2006)</u> <u>Fukai <i>et al.</i> (2015)</u> <u>Gray <i>et al.</i> (2020)</u> <u>LaRocco <i>et al.</i> (2015)</u>
<u>Real-time RT-PCR</u> ☒ ☐ ☐	<u>[1] Testing of samples from FMDV antibody-negative animals (blood and swabs) to rule out pre-clinical/sub-clinical infection</u> <u>[2] Testing of probang samples can be used to confirm FMDV-antibody results</u>	<u>[1] WOAHP <i>Terrestrial Manual</i> describes two methods 3D and 5'UTR and these have different sensitivity and specificity</u> <u>[2] DSp ~100%</u> <u>DSe ~100%</u>	<u>[1] This test purpose is supported by data generated from experimental studies in cattle and other species</u> <u>[2] Further validation data including the use of RT-PCR to detect pre-clinically infected animals arises from FMD outbreaks</u>	<u>[1] See references and Appendix 4: Confirmation of clinical cases</u>	<u>[1] Rapid</u> <u>[2] High analytical and diagnostic sensitivity and specificity</u> <u>[3] Test can be used for all sample types</u> <u>[4] Can adapt testing to high throughput</u>	<u>[1] Test performance dependent upon primers selected</u> <u>[2] requires specific equipment</u> <u>[3] Cross-contamination during sample preparation can lead to false positive results</u> <u>[4] Sequence variability can generate false negative results (particularly for the 5'UTR test)</u> <u>[5] Pan-serotypic real time RT-PCRs in the WOAHP <i>Terrestrial Manual</i> do not give details of serotype</u>	<u>Callahan <i>et al.</i> (2002)</u> <u>King <i>et al.</i> (2006)</u> <u>Moniwa <i>et al.</i> (2007)</u> <u>Shaw <i>et al.</i> (2004)</u> <u>Shaw <i>et al.</i> (2007)</u>
<u>RT-PCR</u> ☒ ☐ ☐	<u>[1] Testing of samples from FMDV antibody-negative animals (blood and swabs) to rule</u>	<u>[1] DSe 97%</u> <u>[2] DSp ~100%</u> <u>[3] Analytical sensitivity reported</u>	<u>Limited validation data is available for this purpose</u>	<u>[1] See references and Appendix 4: Confirmation of clinical cases</u>	<u>[1] Alternative method when there is no real time RT PCR equipment available</u>	<u>[1] Test performance dependent upon primers selected</u> <u>[2] Running a gel increases the risk of cross-contamination</u>	<u>Reid <i>et al.</i> (2000)</u> <u>Reid <i>et al.</i> (2003)</u> <u>Vangrype & De Clercq (1996)</u>

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
	out pre-clinical/sub-clinical infection [2] Testing of probang samples can be used to confirm FMDV-antibody results	to be less than 1 pfu					
NSP Ab ELISA ☒ ☒ ☐	[1] Sera collected to rule out historical exposure to FMDV	[1] DSe between 95% and 100% [2] DSp between 98% and 100% [3] Various NSP ELISA kits exist and these will have different DSe and DSp	[1] Test validated for cattle, sheep, goats and pigs [2] Samples from vaccinated and/or infected animals	[1] See references and Appendix 1: Population freedom from infection	[1] High diagnostic sensitivity in non-vaccinated populations [2] A single test covers all FMDV serotypes [3] Tests are usually appropriate for all susceptible species [4] Easy to perform, suitable for large-scale application or automation [5] Simple-to-use commercial kits are available [6] Ideal for surveillance, control, and eradication	[1] Specificity is < 100% therefore, confirmatory testing may be required [2] Lower specificity is expected when animals have previously been vaccinated [3] Test will only generate a positive result for historical exposure to FMDV >7–10 days after infection. If more recent infection cannot be ruled out, other (molecular or culture) test methods may be required.	Bergmann <i>et al.</i> (2003) Brocchi <i>et al.</i> (2006) Browning <i>et al.</i> (2020) Bruderer <i>et al.</i> (2004) Campos <i>et al.</i> (2008) Lu <i>et al.</i> (2007) Malirat <i>et al.</i> (1998) Paton <i>et al.</i> (2006) Sharma <i>et al.</i> (2014) Sorensen <i>et al.</i> (2005)
SP Ab ELISA ☒ ☒ ☐	[1] Sera collected to rule out historical exposure to FMDV	[1] DSe between 93% and 100%	[1] Test validated for cattle, sheep, goats and pigs	[1] See references and Appendix 3: Contribute to eradication policies	[1] Simple-to-use commercial kits are available [2] SP-abs appear early after infection or vaccination so SP-ELISAs have higher diagnostic sensitivity compared with NSP ELISAs [3] Tests are usually appropriate for all susceptible species [4] Easy to perform, suitable for large-scale application or automation	[1] SP Ab ELISAs cannot be used if vaccines are used as the test cannot differentiate between antibodies generated after vaccination or infection [2] Test will only generate a positive result for historical exposure to FMDV >7–10 days after infection. If more recent infection cannot be ruled out, other (molecular or culture) test methods may be required. [3] Often cross-reactivity across serotype	Hamblin <i>et al.</i> (1986) Hamblin <i>et al.</i> (1987) Ludi <i>et al.</i> (2022) Mackay <i>et al.</i> (2001) Paiba <i>et al.</i> (2004)
VNT ☒ ☒ ☐	Sera collected to rule out historical exposure to FMDV	[1] DSp≈100% [2] DSe≈100% [3] Test performance is influenced by the specific antigen used [4] Test conditions	[1] Test validated for cattle, sheep, goats and pigs	[1] See references and Appendix 3: Contribute to eradication policies	[1] High sensitivity and specificity	[1] VNT test cannot be used if vaccines are used as the test cannot differentiate between antibodies generated after vaccination or infection [2] Test will only generate a positive result for historical exposure to FMDV >7–10 days	Willems <i>et al.</i> (2020) Hamblin <i>et al.</i> (1986)

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
		<u>(cells and condition of cell culture) influence test performance</u>				<u>after infection. If more recent infection cannot be ruled out, other (molecular or culture) test methods may be required.</u> <u>[3] It requires 1–3 days for execution</u> <u>[4] Maintenance and use of live virus and tissue culture cells, thus biocontainment restriction procedures are required</u>	

NOTE: If a suspect non-negative antibody ELISA is received, probang with PCR testing could be used a complementary test to demonstrate freedom.

Appendix 3
Foot and mouth disease
Intended purpose of test: contribute to eradication policies

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Virus isolation</u> <u>✓✓✓</u>	<u>[1] Vesicular liquid, epithelia, swabs, milk, probangs, sera*, blood*</u> <u>*FMD viraemia is transient (~7 days), therefore use of blood sampling should consider the likely pathogenic cycle of infection</u>	<u>[1] Considered to be the gold standard method</u> <u>[2] Test performance is dependent upon the cell line and culture conditions that are used</u> <u>[3] For appropriate samples collected from acutely infected animals, DSe and DSp can approach 100% using highly sensitive cell lines (example LFBKαVβ6)</u>	<u>[1] Samples collected from suspected/confirmed cases and after experimental infection</u>	<u>[1] See references</u>	<u>[1] Generates isolates that are useful for subsequent studies for virus characterisation including vaccine selection</u>	<u>[1] Needs a confirmatory testing to confirm the agent causing CPE is FMDV (e.g., Ag-ELISA or sequencing)</u> <u>[2] Material submitted for testing must be in good condition requiring that a cold chain is maintained from sample collection to the laboratory</u> <u>[3] Testing can be impacted by gross bacterial/fungal contamination, despite the use of antibiotics in the cell culture media</u> <u>[4] Time-consuming and specialist facilities are needed (with appropriate biocontainment)</u>	<u>Ferris <i>et al.</i> (2006)</u> <u>Fukai <i>et al.</i> (2015)</u> <u>Gray <i>et al.</i> (2020)</u> <u>LaRocco <i>et al.</i> (2015)</u>
<u>Antigen detection</u> <u>ELISA</u> <u>✓✓✓</u>	<u>[1] Field samples can be directly tested without VI if suitable (i.e. epithelia, vesicular fluid)</u> <u>[2] Other sample types may require virus isolation first</u>	<u>[1] DSe 83% virus-positive epithelia where viral loads are high in samples collected from acute cases, but lower where sampling focusses on convalescent animals</u> <u>[2] ELISA formats can differ, using polyclonal, monoclonal and/or recombinant integrin. The selection of these reagents impacts on the DSe and DSp of the test</u>	<u>[1] Samples collected from suspected/confirmed cases and after experimental infection</u>	<u>[1] See references</u>	<u>[1] Possibility of virus typing when the laboratory lacks equipment for PCR and sequencing</u> <u>[2] Can be used to serotype isolates generated by culture methods</u> <u>[3] Commercial kit version of this test are available</u>	<u>[1] Lower analytical sensitivity in relation to other methods available (e.g. RT-PCR)</u> <u>[2] DSe is impacted by the ability of the selected antibody reagents to detect diverse FMDV lineages</u> <u>[3] Direct testing of clinical samples is only applicable to epithelial suspensions and fluids</u>	<u>Ferris <i>et al.</i> 2011</u> <u>Grazioli <i>et al.</i> 2020</u> <u>Roeder & Le Blanc Smith (1987)</u>

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>LFD</u> <u>✓✓✓</u>	[1] Saliva, tissue, vesicular fluid and cultivated virus [2] Other sample types such as blood and serum may also be appropriate	[1] DSe 87–99% [2] DSp 99–100%	[1] Most validation data focusses on samples from cattle or pigs	[1] See references [2] Further validation studies are currently underway	[1] Rapid and simple to perform [2] No need for laboratory equipment [3] FMDV positive LFDs can be submitted for molecular analyses	[1] As per antigen detection ELISA	Ferris <i>et al.</i> (2009) Ferris <i>et al.</i> (2012) Oem <i>et al.</i> (2009)
<u>Real-time RT-PCR</u> <u>✓✓✓</u>	[1] All samples collected from acutely infected animals (vesicular liquid, epithelia, sera, blood, milk) [2] Environmental samples (including air) [3] Probang samples from convalescent animals	[1] WOA <i>Terrestrial Manual</i> describes two methods 3D and 5'UTR and these have different sensitivity and specificity [2] DSp ~100% DSe ~100%	[1] Samples collected from suspected/confirmed cases and after experimental infection	[1] See references	[1] Rapid [2] High analytical and diagnostic sensitivity and specificity [3] Test can be used for all sample types [4] Can adapt testing to automated platforms to achieve high throughput	[1] Test performance dependent upon primers selected [2] Requires specific equipment [3] Cross-contamination during sample preparation can lead to false positive results [4] Sequence variability can generate false negative results (particularly for the 5'UTR test) [5] Pan-serotypic real time RT-PCRs in the WOA <i>Terrestrial Manual</i> do not give details of serotype	Callahan <i>et al.</i> (2002) King <i>et al.</i> (2006) Moriwa <i>et al.</i> (2007) Reid <i>et al.</i> (2009) Shaw <i>et al.</i> (2004) Shaw <i>et al.</i> (2007)
<u>RT-PCR</u> <u>✓✓✓</u>	[1] All samples collected from acutely infected animals (vesicular liquid, epithelia, sera, blood, milk)	[1] DSe 97% [2] DSp ~100% [3] Analytical sensitivity reported to be less than 1 pfu	[1] Samples collected from suspected/confirmed cases and after experimental infection		[1] Alternative when there is no real time RT-PCR equipment available	[1] Test performance dependent upon primers selected [2] Running a gel increases the risk of cross-contamination	Reid <i>et al.</i> (2000) Reid <i>et al.</i> (2003) Vangrype & De Clercq (1996)
<u>NSP Ab ELISA</u> <u>✓✓✓</u>	[1] Sera	[1] DSe between 95% and 100% [2] DSp between 98% and 100% [3] various NSP ELISA kits exist and these will have different DSe and DSp	[1] Test validated for cattle, sheep, goats and pigs [2] Samples collected from suspected/confirmed cases and after experimental infection	[1] See references	[1] High diagnostic sensitivity in non-vaccinated populations [2] A single test covers all FMDV serotypes [3] Tests are usually appropriate for all susceptible species [4] Easy to perform, suitable for large-scale application or automation [5] Simple-to-use commercial kits are available	[1] Specificity is < 100% therefore, confirmatory testing often required [2] Lower sensitivity should be expected if animals have been previously vaccinated	Bergmann <i>et al.</i> (2003) Brocchi <i>et al.</i> (2006) Bruderer <i>et al.</i> (2004) Campos <i>et al.</i> (2008) Lu <i>et al.</i> (2007) Malirat <i>et al.</i> (1998) Paton <i>et al.</i> (2006) Sharma <i>et al.</i> (2014) Sorensen <i>et al.</i> (2005)

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
					<u>[6] Ideal for surveillance, control, and eradication</u>		
<u>SP Ab ELISA</u> <u>✓✓✓</u>	<u>[1] Sera</u>	<u>[1] DSe between 93% and 100%</u> <u>[2] DSp between 98% and 100%</u> <u>[2] A range of different test formats are available including in-house and commercial assays. The performance of these tests vary.</u>	<u>[1] Test validated for cattle, sheep, goats and pigs</u> <u>[2] Samples collected from suspected/confirmed cases and after experimental infection</u>	<u>[1] See references</u>	<u>[1] Simple-to-use commercial kits are available</u> <u>[2] SP-abs appear early after infection or vaccination so SP-ELISAs have higher diagnostic sensitivity compared with NSP ELISAs</u> <u>[3] Tests are usually appropriate for all susceptible species</u> <u>[4] Easy to perform, suitable for large-scale application or automation</u>	<u>[1] Cannot differentiate between antibodies generated after vaccination or infection</u> <u>[2] Often cross-reactivity across serotype</u>	<u>Browning <i>et al.</i> (2020)</u> <u>Hamblin <i>et al.</i> (1986)</u> <u>Hamblin <i>et al.</i> (1987)</u> <u>Ludi <i>et al.</i> (2022)</u> <u>Mackay <i>et al.</i> (2001)</u> <u>Paiba <i>et al.</i> (2004)</u>
<u>VNT</u> <u>✓✓✓</u>	<u>Sera</u>	<u>[1] DSp≈100%</u> <u>[2] DSe≈100%</u> <u>[3] Test performance is influenced by the specific antigen used</u> <u>[4] Test conditions (cells and condition of cell culture) influence test performance</u>	<u>[1] Test validated for cattle, sheep, goats and pigs</u> <u>[2] Samples collected from suspected/confirmed cases and after experimental infection</u>	<u>[1] See references</u>	<u>[1] High sensitivity and specificity</u>	<u>[1] It requires 1–3 days for execution</u> <u>[2] Maintenance and use of live virus and tissue culture cells, thus biocontainment restriction procedures are required</u>	<u>Willems <i>et al.</i> (2020)</u> <u>Hamblin <i>et al.</i> (1986)</u>

Appendix 4
Foot and mouth disease
Intended purpose of test: confirmation of clinical cases

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Virus isolation</u> ✓✓✓	[1] Vesicular liquid, epithelia, swabs, probangs, sera*, blood* *FMD viraemia is transient (~7 days), therefore use of blood sampling should consider the likely pathogenic cycle of infection	[1] Considered to be the gold standard method [2] Test performance is dependent upon the cell line and culture conditions that are used [3] For appropriate samples collected from acutely infected animals, DSe and DSp can approach 100% using highly sensitive cell lines (example LFBKαVβ6)	[1] Samples collected from suspected/confirmed cases and after experimental infection	[2] See references.	[1] Generates isolates that are useful for subsequent studies for virus characterisation including vaccine selection	[1] Needs a confirmatory testing to confirm the agent causing CPE is FMDV (e.g. Ag-ELISA or sequencing) [2] Material submitted for testing must be in good condition requiring that a cold chain is maintained from sample collection to the laboratory [3] Testing can be impacted by gross bacterial/fungal contamination, despite the use of antibiotics in the cell culture media [4] Time-consuming and specialist facilities are needed (with appropriate biocontainment)	Ferris <i>et al.</i> (2006) Fukai <i>et al.</i> (2015) Gray <i>et al.</i> (2020) LaRocco <i>et al.</i> (2015)
<u>Antigen detection ELISA</u> ✓✓✓	[1] Field samples can be directly tested without VI if suitable (i.e. epithelia, vesicular fluid) [2] Other sample types may require virus isolation first	[1] DSe 83% virus-positive epithelia where viral loads are high in samples collected from acute cases, but lower were sampling focuses on convalescent animals [2] ELISA formats can differ, using polyclonal, monoclonal and recombinant integrin. The selection of these reagents impacts on the DSe and DSp of the test	[1] Samples collected from suspected/confirmed cases and after experimental infection	[1] See references and/or kit inserts	[1] Possibility of virus typing when the laboratory lacks equipment for PCR and sequencing [2] Can be used to serotype isolates generated by culture methods [3] Commercial kit version of this test are available	[1] Lower analytical sensitivity in relation to other methods available (e.g., RT-PCR) [2] DSe is impacted by the ability of the selected antibody reagents to detect diverse FMDV lineages [3] Direct testing of clinical samples is only applicable to epithelial suspensions and fluids	Ferris <i>et al.</i> 2011 Grazioli <i>et al.</i> 2020 Roeder & Le Blanc Smith (1987)
<u>LFD</u> ✓✓✓	[1] Saliva, tissue homogenates, vesicular fluid and cultivated virus	[1] Similar test performance to Ag-ELISA	[1] Samples collected from suspected/confirmed cases and after experimental infection	[1] See references [2] Further validation	[1] Rapid and simple to perform [2] No need for laboratory equipment	As per antigen detection ELISA	Ferris <i>et al.</i> (2009) Ferris <i>et al.</i> (2012) Oem <i>et al.</i> (2009)

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
	<u>[2] Other sample 3types such as blood and serum may also be appropriate</u>	<u>[2] DSe 87–99%</u> <u>[1] DSp 99–100%</u>		<u>studies are currently underway</u>	<u>[3] FMDV positive LFDs can be submitted for molecular analyses</u>		
<u>Real-time RT-PCR</u> <u>✓✓✓</u>	<u>[1] All samples collected from acutely infected animals (vesicular liquid, epithelia, sera, blood, milk)</u> <u>[2] Environmental samples (including air)</u> <u>[3] Probang samples from convalescent animals</u>	<u>[1] WOA <i>Terrestrial Manual</i> describes two methods 3D and 5'UTR and these have different sensitivity and specificity</u> <u>[2] DSp ~100%</u> <u>DSe ~100%</u>	<u>[1] Samples collected from suspected/confirmed cases and after experimental infection</u>	<u>[1] See references</u>	<u>[1] Rapid</u> <u>[2] High analytical and diagnostic sensitivity and specificity</u> <u>[3] Test can be used for all sample types</u> <u>[4] Can adapt testing to automated platforms to achieve high throughput</u>	<u>[1] Test performance dependent upon primers selected</u> <u>[2] Requires specific equipment</u> <u>[3] Cross-contamination during sample preparation can lead to false positive results</u> <u>[4] Sequence variability can generate false negative results (particularly for the 5'UTR test)</u> <u>[5] Panserotypic real time RT-PCRs in the WOA <i>Terrestrial Manual</i> do not give details of serotype</u>	<u>Callahan <i>et al.</i> (2002)</u> <u>King <i>et al.</i> (2006)</u> <u>Moniwa <i>et al.</i> (2007)</u> <u>Shaw <i>et al.</i> (2004)</u> <u>Shaw <i>et al.</i> (2007)</u>
<u>RT-PCR</u> <u>✓✓✓</u>	<u>[1] All samples collected from acutely infected animals (vesicular liquid, epithelia, sera, blood, milk)</u>	<u>[1] DSe 97%</u> <u>[2] DSp ~100%</u> <u>[3] Analytical sensitivity reported to be less than 1 pfu</u>	<u>[1] Samples collected from suspected/confirmed cases and after experimental infection</u>		<u>[1] Alternative when there is no real time RT PCR equipment available</u>	<u>[1] Test performance dependent upon primers selected</u> <u>[2] Running a gel increases the risk of cross-contamination</u>	<u>Reid <i>et al.</i> (2000)</u> <u>Reid <i>et al.</i> (2003)</u> <u>Vangrype & De Clercq (1996)</u>
<u>NSP Ab ELISA</u> <u>✓✓✓</u>	<u>[1] Sera</u>	<u>[1] DSe between 95% and 100%</u> <u>[2] DSp between 98% and 100%</u> <u>[3] various NSP ELISA kits exist and these will have different DSe and DSp</u>	<u>[1] Samples from all susceptible species that have been exposed to FMDV (field and experimental studies)</u> <u>[2] Validation of this purpose includes sera collected from vaccinated and/or infected animals</u>	<u>[1] See references</u>	<u>[1] High diagnostic sensitivity in non-vaccinated populations</u> <u>[2] A single test covers all FMDV serotypes</u> <u>[3] Tests are usually appropriate for all susceptible species</u> <u>[4] Easy to perform, suitable for large-scale application or automation</u> <u>[5] Simple-to-use commercial kits are available</u>	<u>[1] FMDV-specific antibodies arise after infection. Therefore, these tests are only appropriate if infection >7–10 days is suspected.</u> <u>[2] Specificity is < 100% therefore, confirmatory testing is required</u> <u>[3] Lower sensitivity should be expected if animals have been previously vaccinated</u>	<u>Bergmann <i>et al.</i> (2003)</u> <u>Brocchi <i>et al.</i> (2006)</u> <u>Bruderer <i>et al.</i> (2004)</u> <u>Campos <i>et al.</i> (2008)</u> <u>Lu <i>et al.</i> (2007)</u> <u>Malirat <i>et al.</i> (1998)</u> <u>Paton <i>et al.</i> (2006)</u> <u>Sharma <i>et al.</i> (2014)</u> <u>Sorensen <i>et al.</i> (2005)</u>
<u>SP Ab ELISA</u> <u>✓✓✓</u>	<u>[1] Sera</u>	<u>[1] DSe between 93% and 100%</u> <u>[2] DSp between 98%</u>	<u>[1] Samples from all susceptible species that have been exposed to</u>	<u>[1] See references</u>	<u>[1] Simple-to-use commercial kits are available</u> <u>[2] SP-abs appear early after</u>	<u>[1] FMDV-specific antibodies arise after infection. Therefore, these tests are</u>	<u>Browning <i>et al.</i> (2020)</u> <u>Hamblin <i>et al.</i> (1986)</u> <u>Hamblin <i>et al.</i> (1987)</u>

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
		and 100% [2] A range of different test formats are available including in-house and commercial assays. The performance of these tests vary	FMDV (field and experimental studies)		infection or vaccination so SP-ELISAs have higher diagnostic sensitivity compared with NSP ELISAs [3] Tests are usually appropriate for all susceptible species [4] Easy to perform, suitable for large-scale application or automation	only appropriate if infection >7–10 days is suspected. [2] Cannot differentiate between antibodies generated after vaccination or infection [3] Test exhibit cross-reactivity across FMDV serotypes requiring that further sampling (such as with a probang) may be required to verify the serotype present	Ludi <i>et al.</i> (2022) Mackay <i>et al.</i> (2001) Paiba <i>et al.</i> (2004)
VNT ✓✓✓	Sera	[1] DSP≈100% [2] DSe≈100% [3] Test performance is influenced by the specific antigen used [4] Test conditions (cells and condition of cell culture) influence test performance	[1] Samples from all susceptible species that have been exposed to FMDV (field and experimental studies)	[1] See references	[1] High sensitivity and specificity	[1] FMDV-specific antibodies arise after infection. Therefore, these tests are only appropriate if infection >7–10 days is suspected. [2] Test requires 1–3 days for execution and maintenance and use of live virus and tissue culture cells, thus biocontainment restriction procedures are required	Willems <i>et al.</i> (2020) Hamblin <i>et al.</i> (1986)

Appendix 5
Foot and mouth disease
Intended purpose of test: prevalence of infection – surveillance

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
Real-time RT-PCR ☒ ☐ ☐	[1] All samples collected from acutely infected animals (vesicular liquid, epithelia, sera, blood, milk) [2] Environmental samples (including air) [3] Probang samples from convalescent animals	[1] WOA <i>Terrestrial Manual</i> describes two methods 3D and 5'UTR and these have different sensitivity and specificity [2] DSp ~100% DSe ~100%	[1] Suspected infected animals	[1] See references and Appendix 4: Confirmation of clinical cases	[1] Rapid [2] High analytical and diagnostic sensitivity and specificity [3] Test can be used for all sample types [4] Can adapt testing to high throughput	[1] Test performance dependent upon primers selected [2] Requires specific equipment [3] Cross-contamination during sample preparation can lead to false positive results [4] Sequence variability can generate false negative results (particularly for the 5'UTR test) [5] Pan-serotypic real time RT-PCRs in the WOA <i>Terrestrial Manual</i> do not give details of serotype	Armson <i>et al.</i> (2020) Callahan <i>et al.</i> (2002) King <i>et al.</i> (2006) Moriwa <i>et al.</i> (2007) Shaw <i>et al.</i> (2004) Shaw <i>et al.</i> (2007)
RT-PCR ☒ ☐ ☐	[1] All samples collected from acutely infected animals (vesicular liquid, epithelia, sera, blood, milk)	[1] DSe 97% [2] DSp ~100% [3] Analytical sensitivity reported to be less than 1 pfu	[1] Samples from epithelia collection of different serotypes [2] 5 negative samples	[1] See Appendix 4: Confirmation of clinical cases	[1] Alternative when there is no real time RT-PCR equipment availability	[1] Test performance dependent upon primers selected [2] Running a gel increases the risk of cross-contamination	Reid <i>et al.</i> (2000) Reid <i>et al.</i> (2003) Vangrisperre & De Clercq (1996)
NSP Ab ELISA ☒ ☒ ☒	[1] Sera	[1] DSe between 95% and 100% [2] DSp between 98% and 100% [3] various NSP ELISA kits exist and these will have different DSe and DSp	[1] Cattle, sheep, goats and pigs [2] samples collected as part of sero-surveillance studies [3] Samples from vaccinated and/or infected animals	[1] See references [2] Available from WOA Ref. Lab.	[1] High diagnostic sensitivity in non-vaccinated populations [2] A single test covers all FMDV serotypes [3] Tests are usually appropriate for all susceptible species [4] Easy to perform, suitable for large-scale application or automation [5] Simple-to-use commercial kits are available [6] Ideal for surveillance, control, and eradication	[1] Specificity is < 100% therefore, confirmatory testing is required [2] Lower specificity should be expected if animals have been previously vaccinated	Bergmann <i>et al.</i> (2003) Brocchi <i>et al.</i> (2006) Browning <i>et al.</i> (2020) Bruderer <i>et al.</i> (2004) Campos <i>et al.</i> (2008) Lu <i>et al.</i> (2007) Malirat <i>et al.</i> (1998) Paton <i>et al.</i> (2006) Sharma <i>et al.</i> (2014) Sorensen <i>et al.</i> (2005)
SP Ab ELISA ☒ ☒ ☐	[1] Sera	[1] DSe between 93% and 100%	[1] See reference	[1] See Appendix 3: Contribute to eradication policies [2] Available	[1] Simple-to-use commercial kits are available [2] SP-abs appear early after infection or vaccination so SP-ELISAs have higher diagnostic sensitivity compared with NSP	[1] SP Ab ELISAs cannot be used if vaccines are used. Cannot differentiate between antibodies generated after vaccination or infection [2] Less sensitive than RT-PCR	Hamblin <i>et al.</i> (1986) Hamblin <i>et al.</i> (1987) Ludi <i>et al.</i> (2022) Mackay <i>et al.</i> (2001) Paiba <i>et al.</i> (2004)

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
				from WOAH Ref. Lab.	<u>ELISAs</u> <u>[3] Tests are usually appropriate for all susceptible species</u> <u>[4] Easy to perform, suitable for large-scale application or automation</u>	<u>[3] Tests often suffer from cross-reactivity across serotype which limits their use for serotype-specific surveillance in countries/regions where multiple serotypes co-circulate</u> <u>[4] SP Ab ELISAs cannot be used if vaccines are used as the test cannot differentiate between antibodies generated after vaccination or infection</u>	
<u>VNT</u> ☒ ☒ ☐	<u>Sera</u>	<u>[1] DSp≈100%</u> <u>[2] DSe≈100%</u> <u>[3] Test performance is influenced by the specific antigen used</u> <u>[4] Test conditions (cells and condition of cell culture) influence test performance</u>	<u>[1] Susceptible animals</u>	<u>[1] See Appendix 3: Contribute to eradication policies</u> <u>[2] Recognised as the gold standard method and consequently initial test validation was not performed</u>	<u>[1] High sensitivity and specificity</u>	<u>[1] It requires 1–3 days for execution</u> <u>[2] Maintenance and use of live virus and tissue culture cells, thus biocontainment restriction procedures are required</u> <u>[3] VNT cannot be used if vaccines are used as the test cannot differentiate between antibodies generated after vaccination or infection</u>	<u>Willems <i>et al.</i> (2020)</u> <u>Hamblin <i>et al.</i> (1986)</u>

Appendix 6

Foot and mouth disease

Intended purpose of test: Immune status in individual animals or populations post-vaccination

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>SP Ab ELISA</u> <u>✓✓✓</u>	<u>[1] Sera from vaccinated animals</u> <u>[2] Sera collected for the purposes of vaccine control</u>	<u>[1] DSe ~100%</u> <u>DSp ~87%</u>	<u>[1] Validation data mostly available from cattle in controlled infection or vaccination studies</u>	<u>[1] See references and Appendix 3: Contribute to eradication policies</u>	<u>[1] Tests are simple-to use</u> <u>[2] Where extensive validation has been undertaken (such as in South America), SP-ELISA testing of vaccinated animals correlates with PGP results avoiding the requirement for challenge tests to assess FMD vaccine quality</u>	<u>[1] Tests suffer from poor inter-serotype specificity which can limit their use when multivalent FMD vaccines are used.</u> <u>[2] Estimating protective responses using SP-ELISA serology is not as precise as VNT</u>	<u>Barnett <i>et al.</i> (2003)</u> <u>Goris <i>et al.</i> (2008)</u> <u>Gubbins <i>et al.</i> (2022)</u> <u>Maradei <i>et al.</i> (2008)</u> <u>Periolo <i>et al.</i> (1993)</u>
<u>VNT TEST</u> <u>✓✓✓</u>	<u>[1] Sera from vaccinated animals</u>	<u>[1] DSp~100%</u> <u>[2] DSe~100%</u> <u>[3] Test performance is influenced by the specific viral antigen used</u> <u>[4] Test conditions (cells and condition of cell culture) influence test performance</u>	<u>[1] Samples from vaccinated animals (including potency tests)</u>	<u>[1] See references</u>	<u>[1] High sensitivity and specificity compared with SP-ELISA</u> <u>[2] Test measures neutralising antibodies which are important correlates of protection</u>	<u>[1] Test requires 1–3 days for execution</u> <u>[2] Maintenance and use of live virus and tissue culture cells, thus biocontainment restriction procedures are required</u>	<u>Gubbins <i>et al.</i>, (2022)</u> <u>Hamblin <i>et al.</i> (1986)</u> <u>Willems <i>et al.</i> (2020)</u>

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CHAPTER 3.1.11.
LEISHMANIOSIS

SUMMARY

Description and importance of the disease: *Leishmaniosis is not a single entity but comprises a variety of syndromes caused primarily by at least 20 Leishmania species affecting humans, transmitted by phlebotomine sand flies belonging to the genera Phlebotomus and Lutzomyia. Leishmaniosis is associated with and limited by the geographic distribution of its sand fly vectors. Fifteen nosogeographical entities have been classified worldwide, of which 13 have an established or putative zoonotic nature. In recent years, the number of regions becoming Leishmania-endemic has grown significantly, accompanied by an increased number of animal and human cases.*

In humans, the clinical spectrum ranges from asymptomatic infections to those with high mortality, with three distinct forms being classically described: visceral (VL), cutaneous (CL) and mucocutaneous (MCL). Dogs are commonly affected by L. infantum and L. chagasi (now regarded as synonyms), which causes a chronic viscero-cutaneous disease in this host (canine leishmaniosis, CanL). ~~Asymptomatic~~ Subclinical infection in dogs is widespread and contributes to maintaining the long-term presence of the parasite in endemic regions. The clinical appearance and evolution of leishmaniosis is a consequence of complex interactions between the parasite and host immune response. The outcome of infection depends on the ability of host macrophages to effectively destroy the parasite.

Detection of the agent: *When clinical signs and characteristic lesions are present in affected humans and animals, the demonstration of the parasites in stained smears of splenic, bone marrow and lymph node aspirates, of skin scrapings, and in tissue biopsies, gives a positive diagnosis. If the infection is low grade, detection of parasites is possible only by attempting in-vitro isolation or by polymerase chain reaction (PCR). As there are very few morphological differences among various species, the identification of any isolated Leishmania organism relies on biochemical and/or molecular methods. Several centres throughout the world are presently using isoenzyme and DNA characterisation to identify the agent.*

Serological tests: *Serology is the preferred method for diagnosis of VL and CanL, even during the early stages of the disease. In subclinical forms, seropositive cases are confirmed by parasitological diagnosis or PCR. Serology is of less value for CL and MCL. Of the several serological techniques available, the indirect fluorescent antibody test and the enzyme-linked immunosorbent assay are the most suitable. Crude antigens for serodiagnostic tests can be prepared in the laboratory from cultured parasites. Tests based on recombinant antigens may also be used and have a high specificity although can be less sensitive.*

The rapid immunochromatographic assay is easy to carry out and can be performed in veterinary clinics, but has lower diagnostic efficiency than the indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA).

Requirements for vaccines: *There is no effective vaccine available worldwide at present for use in dogs or humans. A number of vaccines for use in animals are under evaluation, and four have been*

authorised for use in dogs (two in Brazil and two in Europe). Beyond other issues to be evaluated, the use of these vaccines is posing present and future challenges to the fields of diagnosis, epidemiology and surveillance of the parasite, especially in countries where the parasite does not occur. Leishmanin skin test antigen is no longer available worldwide and lacks standardisation.

A. INTRODUCTION

Description and impact of the disease: Leishmaniosis comprises a variety of syndromes caused by members of the protozoan parasite *Leishmania* (Kinetoplastida: Trypanosomatidae), which are transmitted to mammal hosts by the bite of infected phlebotomine sand flies belonging to the genera *Phlebotomus* (Old World)¹ and *Lutzomyia* (New World). The disease results from the multiplication of amastigote forms in macrophages of the reticuloendothelial system. The epidemiology and clinical manifestations of the diseases are largely diverse, being usually grouped into two main entities: zoonotic leishmanioses, where domestic or wild animal reservoirs are involved in the transmission cycle and humans play a role as an accidental host, and anthroponotic leishmanioses, where humans are the sole reservoir and source of the vector's infection (Esch & Petersen, 2013). T lymphocytes and cytokines, play a crucial role in determining whether infection evolves toward a protective immune status or a progressive and manifest disease. In inbred mouse models, two different T helper (Th) cell subsets are involved, termed Th1 and Th2, which differ in their profile of secreted cytokines by a polarised activation; the Th1 response confers protection, whereas the Th2 response renders the host susceptible to infection. However, in human patients and dogs with clinically apparent infections, Th1 and Th2 type responses are not characteristically polarised, as both activating (e.g. interferon gamma, interleukin 12) and suppressive cytokines (e.g. interleukin 10, interleukin 13, interleukin 4, TGF beta) are detected (Murray *et al.*, 2005).

Various forms of clinical manifestations of human leishmaniosis have been described and divided into three major clinical entities: visceral leishmaniosis (VL (synonyms: leishmaniasis, kala azar), cutaneous leishmaniosis (localised, diffuse or disseminated CL, [visceral form], oriental sore, uta, pian bois, chiclero's ulcer) and mucocutaneous leishmaniosis (MCL, [cutaneous form], espundia) (World Health Organization [WHO], 2010). The diseases are mainly zoonoses with two exceptions, that of CL due to *L. tropica* in urban areas of the Middle East, and that of VL due to *L. donovani* in the Indian sub-continent (northern India, Nepal and Bangladesh) and in some parts of Eastern Africa (e.g. Ethiopia and Sudan). Canine leishmaniosis (CanL) [mucocutaneous form] is a chronic viscero-cutaneous disease caused by *L. infantum*, for which the dog acts as the source reservoir. Resistant asymptomatic dogs may exceed >50% of the infected canine population. Typical external signs recorded in susceptible dogs that evolve towards full-blown disease are lymph node enlargement vector-borne parasitic disease caused by protozoa of the genus *Leishmania*. Transmission occurs through the bite of infected female phlebotomine sand flies, primarily of the genera *Phlebotomus* (Africa and Eurasia) and *Lutzomyia* (Americas). The parasite multiplies as intracellular amastigotes within host macrophages, leading to clinical syndromes of variable severity depending on the host immune response and *Leishmania* species involved. Dogs, which serve as the main domestic reservoir for zoonotic *L. infantum*, frequently develop overt disease. Clinical signs include generalised lymphadenopathy, weight loss, exfoliative dermatitis, ulcerative or alopecic skin lesions, ocular abnormalities, and onychogryphosis, alopecia, ulcers and ocular alterations. Feline leishmaniosis (FeL) caused by *L. infantum* appears to be an emerging feline disease; in the past two decades it has been more and more frequently (Solano-Gallego *et al.*, 2009). Gross pathological findings may include splenomegaly, hepatomegaly, and lymphadenomegaly. Although less common, FeL is increasingly reported in endemic areas and sporadically also seen in non-endemic areas in re-homed cats (typically presents with cutaneous or mucocutaneous lesions (Pennisi *et al.*, 2015; Richter *et al.*, 2014; Rüfenacht *et al.*, 2005). Sporadic tegumentary cases have been reported in a variety of wild and domestic species, such as wild canids, equids and cattle (Gramiccia *et al.*, 2011). Likewise, wild canid (wolf, fox and jackal) populations and lagomorphs, can show similar infection rates act as in dogs, but the prevalence of progressive clinical signs in these species is substantially lower and thus they may not be as infectious as dog populations to supporting a primary transmission cycle. In an outbreak of visceral leishmaniosis in Spain, reservoirs or incidental hosts. *Leishmania infantum* has been detected in hares (*Lepus granatensis*) and rabbits (*Oryctolagus cuniculus*) were described as a reservoir (Jimenez *et al.*, 2014; during human outbreaks in Spain (Molina *et al.*, 2012).

Causal pathogen: Approximately 20 In humans, three clinical forms are recognised *Leishmania* species are agents of human leishmanioses. In the New World, tegumentary forms are caused by *L.*: visceral leishmaniosis (VL), cutaneous leishmaniosis (CL), and mucocutaneous leishmaniosis (MCL). VL is fatal if untreated; CL causes ulcerative skin lesions that may self-resolve; and MCL leads to disfiguring mucosal damage (WHO, 2010). The disease occurs in tropical, subtropical, and some temperate areas, with seasonal transmission in cooler climates and continuous transmission in warmer regions. panamericanis, *L. shawi*, *L. naiffi*, *L. lainsoni*, *L. lindenbergi*, *peruviana*, *L. mexicana*, *L. venezuelensis* and *L. amazonensis*; visceral and, more rarely, cutaneous forms, are caused by *L. infantum*. In the Old World, cutaneous forms are caused by *L. tropica*, *L. major* and *L. aethiopica*; visceral and, more rarely, cutaneous forms, are caused by *L. donovani*. *Leishmania infantum* and

¹ In this chapter, the term 'New World' refers to the Americas, and the term 'Old World' refers to Africa, Asia and Europe (WHO, 2010).

L. chagasi have been found to be identical by genotyping and should be regarded as synonyms (Kuhls *et al.*, 2011). In addition, the taxonomic position of other *Leishmania* agents of tegumentary forms (*L. killicki* in the Old World; *L. pifanoi*, *L. garnhami* and *L. colombiensis* in the New World) is still under discussion. Other *Leishmania* species not pathogenic to humans include New and Old World rodent parasites and an agent of cutaneous leishmaniasis in Australian macropods (Dougall *et al.*, 2009). Dogs are mainly affected by *L. infantum*, but, other *Leishmania* species are found in dogs in the New World (*Leishmania amazonensis*, *L. braziliensis*, *L. mexicana*, *L. venezuelensis*) (Pennisi *et al.*, 2015; Solano-Gallego *et al.*, 2009). *Leishmania tropica* and *L. major* are rarely reported in dogs and are found mainly associated with skin or mucocutaneous lesions (Baneth *et al.*, 2017).

Nature and classification of the pathogen: *Leishmania* spp. are protozoan parasites in the order Kinetoplastida, family Trypanosomatidae. Around 20 species are pathogenic to humans. In Africa and Eurasia, important species include *L. infantum*, *L. donovani*, *L. tropica*, *L. major*, and *L. aethiopica*; in the Americas, *L. braziliensis*, *L. guyanensis*, *L. mexicana*, and *L. amazonensis* are prominent. *L. infantum* and *L. chagasi* are genetically indistinguishable and considered synonymous (Kuhls *et al.*, 2011). The parasite alternates between two main stages: the extracellular promastigote in the vector and the intracellular amastigote in the vertebrate host. Infection occurs when the promastigotes are injected into the host's skin during the sand fly's blood meal. The clinical outcome is strongly influenced by the host's immune response.

Zoonotic risk potential and biosafety and biosecurity requirements: Leishmaniasis is a major zoonosis. Most human cases are zoonotic, with domestic dogs as the principal reservoir of *L. infantum*. However, anthroponotic forms also occur, notably with *L. donovani*-related VL in South Asia and *L. tropica*-related CL in urban settings. As transmission is vector-dependent and the parasite lacks environmentally ~~Direct contact with infected hosts or handling of biological samples and parasite cultures from these hosts is of low risk, because of the sand fly-borne nature of the infections and the lack of resistant forms in the environment.~~ All direct zoonotic transmission is minimal. Nonetheless, biosafety is important during laboratory work. Laboratory manipulations with *Leishmania* spp. should be performed ~~at an~~ with appropriate biosafety and containment ~~level procedures as determined by biorisk biological risk analysis as described in (see Chapter 1.1.4 Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities).~~ Prophylactic vaccination of laboratory personnel is not required.

Differential diagnosis: In dogs, the most common leishmaniasis clinical signs may be confounded with ~~resemble other vector-borne diseases such as ehrlichiosis, babesiosis, or dirofilariosis, as well as systemic fungal infections and vector-borne or intestinal helminthiases autoimmune dermatoses.~~

Role of the vector: The phlebotomine vectors of leishmaniasis are, in some cases, only permissive to the complete development of the species of *Leishmania* that they transmit in nature, while in other instances they can transmit more than one species of *Leishmania* (Bates, 2007). There are emerging species of *Leishmania* infecting humans and animals for which the vector has not been identified. There are some 500 known phlebotomine species, but only about 30 have been found to transmit leishmaniasis. Only the female sand fly transmits the parasites (WHO, 2010). In temperate areas the transmission of *Leishmania* occurs only during warm months, coinciding with the activity period of adult sand flies, but due to the variable and lengthy pre-patent period, seasonal variation in the disease incidence in dogs and humans ~~cats, leishmaniasis may not be apparent.~~ By contrast, in warmer regions such as much of endemic Brazil, sand fly activity and human and canine transmission of zoonotic VL is year-round. Temperature affects the development time and overwintering of sand flies and the extrinsic incubation period, which is likely to be reflected in their duration of infectiousness mimic neoplastic, infectious, or immune-mediated skin disorders (Pennisi *et al.*, 2015). In humans, differential diagnoses include tuberculosis and leprosy, especially in cutaneous or mucosal presentations.

B. DIAGNOSTIC TECHNIQUES

Canine leishmaniasis (CanL) diagnosis must be based on an integrated approach that takes into account signalling, medical history, physical findings, clinical and pathological changes, and results of diagnostic tests. The diagnostic methods currently available are described below.

The list and fitness of each test for different purposes are given in Table 1.

Table 1. Test methods available for diagnosis of canine leishmaniosis 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
Detection of the agent ^(a-f)						
Cytological examination	□□□	□□□	□□□	☒ ☒ ☒ ☒ ☒ ☐	□□□	□□□
Histological examination	□□□	□□□	□□□	☒ ☒ ☐	□□□	
Isolation in culture	□□□	☒ □□	□□□	☒ ☒ ☐	□□□	
Molecular methods	☒ ☒ ☐	☒ ☒ ☒	☒ ☐ ☐ □□□	☒ ☒ ☒ ☒ ☒ ☐	☒ ☒ ☐	
Detection of immune response						
IFAT	☒ ☒ ☒	☒ ☒ ☐	☒ ☒ ☒ □□□	☒ ☒ ☐	☒ ☒ ☒	☒ ☐ ☐ □□□
ELISA	☒ ☒ ☒	☒ ☒ ☐	☒ ☒ ☒ □□□	☒ ☒ ☐	☒ ☒ ☒	☒ ☐ ☐ □□□
Direct agglutination test	☒ ☐ ☐ ☒ ☒ ☐	☒ ☐ ☐ ☒ ☒ ☐	□□□	☒ ☒ ☐	☒ ☒ ☐	□□□
Rapid immuno-chromatographic assay	□□□	□□□	□□□	☒ ☒ ☐	☐ ☐ ☐ ☒ ☐ ☐	□□□

Key: ☒☒☒ = recommended for this purpose; ☒☒☐ = recommended but has limitations;

☒☐☐ = suitable in very limited circumstances; □□□ = not appropriate for this purpose.

PCR = polymerase chain reaction; IFAT = Indirect fluorescent antibody test; ELISA = enzyme-linked immunosorbent assay.

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

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

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

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

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

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

Although diseases caused by *Leishmania* spp. are well-documented in dogs and humans, their occurrence in cats has historically received less attention. Felines were once thought to be more resistant to *Leishmania* infections; however, numerous studies over recent decades have established that cats are indeed susceptible and capable of transmitting the parasite to sand fly vectors. Despite a growing number of case reports, there is still a notable absence of comprehensive field studies addressing clinical treatment and management. These developments underscore the significance of feline leishmaniosis (FeL) in veterinary medicine and its relevance to the One Health perspective on leishmaniosis. FeL generally follows a chronic and progressive course. Common clinical signs include skin and mucocutaneous lesions – especially ulcers and nodules on the head and limbs – as well as lymph node enlargement. Uveitis is a frequently observed eye lesion, while oral nodules may appear on the tongue or gums. Recently, unusual cases involving respiratory disease, chronic nasal discharge, and obstructive masses have been described. Other rare manifestations include chronic diarrhoea, mastitis, and panniculitis, all confirmed to involve *L. infantum*.

1. Diagnostic objectives for feline leishmaniosis (FeL)

The primary goal of diagnosis is to confirm whether *Leishmania* infantum is the cause of illness in cats showing clinical signs or laboratory abnormalities consistent with FeL. Quantitative serological tests can support diagnosis when high antibody levels – significantly above the established cut-off – are present in cats with relevant exposure risk and compatible clinical signs. Nonetheless, serology alone is insufficient, and further confirmation through cytological examination is essential. Cytology of affected tissues (e.g. skin, mucosa, lymph nodes, spleen, or lesions found through imaging or endoscopy) can reveal characteristic changes such as lymphoid hyperplasia or inflammatory patterns (neutrophilic, macrophagic, or lymphoplasmacytic). The direct observation of *Leishmania* amastigotes is considered definitive for infection. Cytology also helps identify potential coexisting conditions. Depending on the case, histopathology and immunohistochemical staining for *Leishmania* may be necessary. In cats that test negative or weakly positive for antibodies and do not show tissue lesions, polymerase chain reaction (PCR) or cytological evaluation of bone marrow, lymph nodes, or spleen can help detect the parasite. Importantly, any concurrent diseases must be considered to determine whether *L. infantum* is the actual cause of clinical signs or merely an incidental infection. Currently, there are no validated rapid diagnostic (point-of-care) tests for detecting anti-*Leishmania* antibodies in cats.

FeL diagnosis must be based on an integrated approach that takes into account signalling, medical history, physical findings, clinical and pathological changes, and results of diagnostic tests. The diagnostic methods currently available are described below.

The list and fitness of each test for different purposes are given in Table 2.

Table 2. Test methods available for diagnosis of feline leishmaniosis and their purpose

Method	Purpose					
	<u>Population freedom from infection</u>	<u>Individual animal freedom from infection prior to movement</u>	<u>Contribute to eradication policies^(a)</u>	<u>Confirmation of clinical cases^(a)</u>	<u>Prevalence of infection – surveillance^(a)</u>	<u>Immune status in individual animals or populations post-vaccination^(a)</u>
<u>Detection of the agent^(b)</u>						
<u>Cytological examination</u>	☐ ☐ ☐	☐ ☐ ☐	☐ ☐ ☐	☒ ☒ ☐	☐ ☐ ☐	☐ ☐ ☐
<u>Histological examination</u>	☐ ☐ ☐	☐ ☐ ☐		☒ ☒ ☐	☐ ☐ ☐	
<u>Isolation in culture</u>	☐ ☐ ☐	☐ ☐ ☐		☒ ☐ ☐	☐ ☐ ☐	
<u>Molecular methods</u>	☐ ☐ ☐	☐ ☐ ☐		☒ ☒ ☒	☒ ☒ ☐	
<u>IFAT</u>	☐ ☐ ☐	☐ ☐ ☐	☐ ☐ ☐	☒ ☒ ☐	☒ ☒ ☐	☒ ☐ ☐
<u>ELISA</u>	☐ ☐ ☐	☐ ☐ ☐		☒ ☒ ☐	☒ ☒ ☐	☒ ☐ ☐

Key: ☒☒☒ = recommended for this purpose; ☒☒☐ = recommended but has limitations;

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

PCR = polymerase chain reaction; IFAT = Indirect fluorescent antibody test; ELISA = enzyme-linked immunosorbent assay.

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

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

1. Detection of the agent

1.1. Cytological examination

Cytological examination allows the presence of amastigotes in macrophages or extracellular fluid to be highlighted after appropriate staining (May Grunwald–Giemsa staining). Amastigote are small intracellular rounded or oval body, 1.5–3 × 2.5–6.5 µm in size, found in vacuoles within the cytoplasm of

the macrophages. There is no free flagellum. The organism has a relatively large nucleus and a kinetoplast consisting of a rod-like body and a dot-like basal body.

The cytological investigation should therefore be performed on the following samples:

- i) Papular, nodular and ulcerative skin lesions: sampling by fine needle aspiration or impression smear; ischemic ulcerative lesions may, however, be negative;
- ii) Bone marrow and lymph nodes in the presence of clinical signs or clinical pathological changes referable to the involvement of parasites (anaemia, lymphadenomegaly, etc.);
- iii) Other locations: biological fluids taken from sites with lesions (e.g. synovial fluid in the case of arthritis/polyarthritis, cerebrospinal fluid in the case of neurological signs, etc.). In the absence of sampling lesions, the organs or tissues in which parasites are most likely to be found are represented, in decreasing order of diagnostic sensitivity, by spleen, bone marrow, lymph node and blood (Mylonakis *et al.*, 2005; Saridomichelakis *et al.*, 2005).

1.1.1. Precautions

Splenic aspiration is not recommended due to the high risk of life-threatening bleeding and should be replaced by bone marrow aspiration, which is safer and diagnostically acceptable.

1.1.2. Interpretation

A cytological diagnosis should be considered positive when amastigotes are clearly visualised within macrophages under high magnification fields; however, confirmation by molecular methods is recommended in equivocal cases.

In the case of a negative cytological result, the material used for cytology can be stored and sent to the laboratory for testing by ~~polymerase chain reaction (PCR)~~ (see Section B.1.4 *Molecular methods – detection of nucleic acids*). Cytological examination is supported by extensive field use and empirical performance data; however, it has not been formally validated in accordance with chapter 1.1.6. The specificity of microscopy for the diagnosis of visceral leishmaniasis is high, but its sensitivity varies according to the sampled tissues: 93–99% for the spleen, 52–85% for bone marrow, and 52–58% for lymph node aspirates. ~~However, splenic aspiration is not recommended, given the very high risk of life-threatening bleeding. Bone marrow aspiration is less sensitive but safer and is currently considered the best method for obtaining a sample of tissue to analyse.~~

1.2. Histological examination

The parasite can be highlighted in sections of lesions stained with haematoxylin-eosin. In association with the parasite, the CanL-compatible alterations can also be highlighted, represented by granulomatous inflammation and/or vasculitis affecting different organs, ischaemic dermatopathies, lymphoplasmacytic dermatitis of the dermo-epithelial junction, lymphoid hyperplasia of the spleen and lymph nodes. The use of histological examination is always advisable when, despite a negative cytological examination, the strong suspicion of CanL persists, especially in the presence of dermatitis and in cutaneous forms characterised by focal lesions.

1.2.1. Interpretation

Histological evidence of *Leishmania* amastigotes or compatible inflammatory changes should be considered supportive but not confirmatory in the absence of direct parasite detection. In such cases, confirmation using specific immunohistochemical labelling or molecular techniques is necessary.

If histological alterations such as those described above are detected in haematoxylin & eosin-stained sections, but without detectable parasites it is advisable to proceed with immunohistochemical staining for *Leishmania* antigens. If this approach is also negative, the biopsy sample can be used for genetic analyses (PCR, sequencing, loop-mediated isothermal amplification) (see Section B.1.4 *Molecular methods – detection of nucleic acids*) (Muller *et al.*, 2003; Roura *et al.*, 1999a).

1.2.2. Validation status

Histological examination is widely used and internationally recognised in diagnostic practice; however, its sensitivity is variable, and confirmatory testing is recommended to support diagnosis in ambiguous cases.

1.3. Isolation in culture

Isolation in culture is the most specific test because the development in culture of vital promastigotes is solely attributable to the genus *Leishmania* in the case of samples taken in endemic areas of the ~~Old World–Africa and Eurasia~~. The parasitological investigation should be performed on the following samples: lymph node aspirate, bone marrow aspirate, skin scrapings and biopsies. In VL, blood can also be used (by taking the buffy coat), with reduced sensitivity. However, isolation in culture has the disadvantage of requiring long execution times and is performed only in specialised laboratories. The choice of the isolation and culture methods will depend on the immediate circumstances and on the technical capability and experience of the laboratory staff (WHO, 2010). Unfortunately, there is still no 'universal' culture medium in which all the different leishmanias will grow easily, and it is almost impossible to predict which medium will be best suited to the growth of a particular isolate of *Leishmania*. Individual laboratories have to find the most suitable medium among biphasic blood agar media and tissue culture media supplemented with fetal calf serum (Evans, 1987). When attempting primary isolation of unknown organisms, a blood agar-based medium should be used – preferably NNN medium (Novy, McNeil and Nicolle), otherwise brain–heart infusion (BHI) agar medium or EMTM (Evans' modified Tobie's medium) should be used. For bulk cultivation of established isolates, suitable media are reported in Section B.1.3 below (see Evans, 1987 for media composition). The organisms from patients with chronic CL and MCL can be very difficult to cultivate. The parasites sometimes die when subcultured, even when the initial isolation is successful. This seems especially common when the initial isolation has been into a rich medium. Often this can be overcome if subcultures are made into less nutritionally rich media, such as NNN, or one of the semisolid media such as 'sloppy Evans' or semisolid Locke blood agar. ~~There are many references available offering new alternative culture media to be used for cultivation of the parasite (Castelli et al., 2014; Santarém et al., 2014). Recent comparative studies (e.g. Castelli et al., 2014) have evaluated the growth performance of Leishmania spp. in conventional versus modified culture media (e.g. RPMI-PY), providing useful evidence for media selection and culture optimisation under different laboratory conditions.~~

Validation status: Isolation in culture is recognised as a reference method for specificity, supported by extensive use and comparative performance data. Although it has not undergone formal validation in accordance with chapter 1.1.6, available experimental evidence – such as that provided by Castelli et al. (2023) – supports its reliability when performed under standardised conditions. Sensitivity remains variable and depends on sample quality, parasite load, and laboratory expertise.

1.4. Molecular methods – detection of nucleic acids

Molecular methods for the detection of *Leishmania* nucleic acids include PCR and real-time PCR. These methods are widely adopted for both diagnosis and species identification, given their high analytical sensitivity and specificity. Molecular assays are particularly useful when conventional parasitological methods such as cytology, histology, or culture are negative or not feasible. PCR methods are available for diagnosis and/or identification of *Leishmania* from different types of human and animal samples. The molecular investigation should be performed on the following samples in decreasing order of sensitivity: bone marrow/lymph node, skin, ~~conjunctiva~~, buffy coat, peripheral blood, ~~conjunctiva~~. In resistant dogs, *Leishmania* inoculation may not be followed by parasite dissemination therefore, in endemic areas, a positive PCR result on a skin sample in the absence of skin lesions does not necessarily mean that the dog is infected and will develop infection (Gradoni, 2002). Similarly bone marrow PCR positives may subsequently test negative (Oliva et al., 2006). It is always better to use fresh or frozen material or material fixed in 95% ethyl alcohol. Formalin-fixed or paraffin-fixed samples can be used but give lower diagnostic yields.

~~Essentially, techniques developed either to identify established isolates of *Leishmania* or to detect organisms from several samples, include: (a) digestion of material with proteinase K and DNA extraction. These steps can be either performed using in-house protocols and reagents, or by commercial kits that are widely available; (b) standard PCR amplification using oligonucleotide sequences (primers) selected from the small subunit rRNA gene (Mathis & Deplazes, 1995), kinetoplast DNA minicircles (Maarten et al., 1992), or other highly repetitive genomic DNA sequences (Bulle et al., 2002; Piarroux et al., 1993); (c) analysis of amplification products by 1–2% agarose gel. PCR is a very sensitive method, especially if the PCR targets are "multi-copy" genomic sequences, those present in high numbers in every single parasite, such as the kinetoplast minicircles DNA (Cortes et al., 2004). Precaution: In resistant dogs, *Leishmania* DNA may be detected in the skin in the absence of disseminated infection; therefore, positive PCR results in skin without lesions should be interpreted with caution (Gradoni, 2002).~~

The three most used techniques are:

i) Conventional or traditional PCR: *Leishmania* DNA is amplified using a pair of primers (complementary base sequences to the target sequence contained in *Leishmania* DNA); (Lachaud *et al.*, 2002; Muller *et al.*, 2003);

ii) Nested PCR: a modification of traditional PCR, more sensitive but less specific, as increasing the number of steps tends to increase the risk of contamination by foreign DNA and, therefore, of false positive results (Fisa *et al.*, 2001; Roura *et al.*, 1999b);

iii) ——— Real-time PCR (quantitative): the use of fluorescent molecules or probes makes it possible to quantify the number of DNA copies present in a biological sample. Real-time PCR has a sensitivity similar to the nested PCR, but if performed with 'closed' systems, it is more specific because the sample undergoes a smaller number of manipulations and is therefore less prone to contamination. Real-time PCR can also provide information useful in the monitoring phase, e.g. number of parasites (Manna *et al.*, 2008).

Studies have also demonstrated the potential of loop-mediated isothermal amplification (LAMP) as a point of care diagnostic technique for CanL particularly in resource limited endemic areas (Nzulu *et al.*, 2019).

1.4.1. Extraction of nucleic acids

DNA should be extracted using validated procedures, either through in-house protocols (e.g. proteinase K digestion followed by phenol-chloroform or silica-based purification) or through commercial kits. The quality and quantity of extracted DNA should be assessed (e.g. spectrophotometrically) to ensure suitability for amplification. Where necessary, internal amplification controls should be included to detect PCR inhibition.

1.4.2. Real-time PCR (quantitative PCR)

Several assays for diagnosing leishmaniasis provide only qualitative results (positive/negative), leveraging real-time PCR to reduce analysis time and contamination risk, and are often used for genotyping. Other assays aim to quantify parasite load through absolute or relative quantification, typically using high-copy targets like 18S rRNA or kDNA minicircle sequences. The accuracy of these assays depends on knowing the copy number of the target per cell and its consistency across species or strains. While kDNA copy number can vary among species or isolates, it remains stable within a strain or during patient monitoring, making real-time PCR based on this target reliable for tracking parasitaemia and relapses. The use of real-time PCR in leishmaniasis diagnosis has led to highly sensitive and specific methods that support timely and appropriate treatment (Galluzzi *et al.*, 2018). Compared with conventional techniques, real-time PCR offers advantages such as automation, rapid processing, and high throughput. However, a universally standardised assay that combines parasite quantification with species genotyping in specific regions is still missing. This is due to the lack of agreement on procedures such as sample collection, DNA extraction, target selection, and normalisation strategies. Given the variability among existing assays, which each have their own strengths and weaknesses, no single method is ideal. A combined strategy using multiple real-time PCR assays may improve both sensitivity and specificity. For initial screening, high-copy targets like kDNA minicircles are preferred due to their low detection limits, though their performance can vary depending on species or subgenus. Sequential or parallel assays may be necessary to detect different taxa and estimate parasite load, followed by more specific real-time PCR assays for species identification. While cost-saving methods like SYBR Green with melting or HRM analysis can be used, they limit multiplexing. In low-parasite samples, a nested PCR step may improve target detection and result reliability. Despite advances, the high cost of real-time PCR platforms and reagents continues to limit its routine use in endemic, low-resource settings, although increasing miniaturisation and affordability, may help expand its adoption.

<u>Pathogen/ target gene</u>	<u>Primer/probe (5'-3')</u>	<u>Concentration</u>	<u>Cycling parameters^(a)</u>
<u>Method 1: Castelli <i>et al.</i>, 2021. GenBank Accession No.: AJ131633 [116 bp]</u>			
<u><i>Leishmania donovani</i> complex /kDNA</u>	<u>Fwd: TGC-GTC-CTG-CGT-ATG-GCA-CC Rev: GGC-TGG-CAT-GCC-CGA-ATA-GCA Probe: TTT-TTC-GCG-AAT-GGG-GGA-TGT-GG</u>	<u>400 nM 300 nM</u>	<u>50 cycles of: 95°C/15 sec and 58°C/60 sec</u>
<u>Method 2: Marfurt <i>et al.</i>, 2003; GenBank Accession No.: Z35271 [120 bp]</u>			

<u><i>Leishmania</i> spp./kDNA</u>	Fwd: CTT-TTC-TGG-TCC-CGC-GGG-TAG-G Rev: CCA-CCT-GGC-CTA-TTT-TAC-ACC-A Probe: TTT-TCG-CAG-AAC-GCC-CCT-ACC-CGC	400 nM 200 nM	50 cycles of: 95°C/15 sec. 58°C/60 sec
Method 3: Wortmann <i>et al.</i> , 2001; GenBank Accession No.: AF291436 [150 bp]			
<u><i>Leishmania</i> spp./18S rRNA</u>	Fwd: AAG-TGC-TTT-CCC-ATC-GCA-ACT Rev: CGC-CGA-AGT-TGA-GGC-AAT-AA Probe: CGT-GTC-GCC-CTG-GAT-GTC-GG	400 nM 200 nM	50 cycles of: 95°C/15 sec. 58°C/60 sec

1.4.3. Conventional PCR

Conventional PCR is a fundamental amplification technique widely used for the detection of *Leishmania* DNA in clinical or entomological samples. It relies on three key cyclic steps: denaturation of the DNA template (typically at 94–95°C) to separate the strands, annealing of sequence-specific primers to their complementary targets (usually between 50–65°C), and extension mediated by a thermostable DNA polymerase (commonly at 72°C). The reaction is typically carried out for 30–40 cycles and may include an initial denaturation step and a final extension phase. Amplified products are subsequently visualised by agarose gel electrophoresis. In the context of leishmaniasis diagnostics, the most commonly used molecular targets in conventional PCR include:

- Method 1-kDNA: a minicircle region of kinetoplast mitochondrial DNA, present in high copy number, enabling highly sensitive detection (amplicon ~120 bp);
- Method 2-ITS-1: the internal transcribed spacer 1 of the ribosomal DNA cluster, used for both detection and species discrimination through restriction fragment length polymorphism (RFLP) analysis (amplicon ~320 bp);
- Method 3-18S rRNA: a highly conserved ribosomal gene, suitable for genus-level detection of *Leishmania* (amplicon ~150 bp).

<u>Pathogen/ target gene</u>	<u>Primer (5'–3')</u>	<u>Concentration</u>	<u>Cycling parameters^(a)</u>
Method 1: Schönian <i>et al.</i>, 2003; GenBank: AF308682; ~320 bp			
<u><i>Leishmania</i> spp./ITS-1</u>	Fwd: CTG-GAT-CAT-TTT-CCG-ATG Rev: TGA-TAC-CAC-TTA-TCG-CAC-TT	400 nM	35 cycles of: 94°C/30 sec. 55°C/30 sec, 72°C/1 min
Method 2: Marfurt <i>et al.</i> , 2003; GenBank: Z35271; 120 bp			
<u><i>Leishmania</i> spp./kDNA</u>	Fwd: CTT-TTC-TGG-TCC-CGC-GGG-TAG-G Rev: CCA-CCT-GGC-CTA-TTT-TAC-ACC-A	400 nM	40 cycles of: 94°C/30 sec. 60°C/30 sec, 72°C/30 sec
Method 3: Wortmann <i>et al.</i> , 2001; GenBank: AF291436; 150 bp			
<u><i>Leishmania</i> spp./18S rRNA</u>	Fwd: AAG-TGC-TTT-CCC-ATC-GCA-ACT Rev: CGC-CGA-AGT-TGA-GGC-AAT-AA	400 nM	40 cycles of: 95°C/15 sec. 58°C/60 sec

1.5. Molecular characterisation [formerly Sections B.3 and B.3.2]

Morphological identification enables identification of *Leishmania* at the genus level, but not at the species or subspecies level. Several techniques may be used to identify the different *Leishmania* species, subspecies or strains. Fifteen recognised *Leishmania* Identification Centres were listed by WHO in 2010.

Different molecular techniques have been developed that allow *Leishmania* characterisation at the species or strain level such as (a) PCR-RFLP analysis, in which the PCR products are digested by appropriate restriction enzymes and the resulting restriction fragment patterns are analysed (Marfurt *et al.*, 2003); (b) multi-locus microsatellite typing (MLMT) (Kuhls *et al.*, 2011) and (c) multi-locus sequence typing (MLST) (Mauricio *et al.*, 2006). In these cases, repeated and polymorphic DNA sequences are targeted, such as ribosomal internal transcribed spacer 1 (ITS1), cysteine protease B, kinetoplast DNA minicircles, surface glycoprotein 63, heat-shock protein 70, mini-exons and microsatellites.

1.6. Isoenzyme characterisation [formerly Section B.3.1.]

Also known as multi-locus enzyme electrophoresis (MLEE), isoenzyme characterisation is the reference method for species identification (WHO, 2010), although this technique requires cultivation of a large number of parasites (5×10^9 – 1×10^{10}). The principles of enzyme electrophoresis are as follows: soluble enzymes are extracted from the organisms grown in media for bulk cultivation (BHI medium, MEM/FCS/EBLB [minimal essential medium/fetal calf serum/Evans' blood lysate broth] medium, Schneider's *Drosophila* medium). A small amount of the extract is then placed in an inert supporting substance, the matrix, containing a buffer at a fixed pH. The matrix is usually starch gel, but it could equally well be absorbent cellulose acetate, acrylamide or agarose. The pH of the buffer in the matrix is usually chosen so that the isoenzymes are negatively charged. A direct current is passed through the matrix carried by the ions in the buffer. When electrophoresis is completed, most proteins will have moved in the matrix towards the anode, depending on the amount of negative charge. If stained at this stage with a general protein stain, many bands will be seen. However, the high substrate and cofactor specificity of enzymes make it possible to stain only these proteins. Hence, the electrophoretic mobility of one particular enzyme can be compared among several organisms. The stained matrix with its collection of stained isoenzyme bands is known as a zymogram. Normally one or more extracts from reference organisms, in which the enzyme banding patterns are well documented, are included in the gel to aid the interpretation of results. Most enzymes used for characterisation purposes are stained by methods incorporating a dehydrogenase reaction. At least 12 enzymes should be examined; organisms showing identical zymograms are classified into zymodemes of a given species.

2. Serological tests

Seroconversion occurs within a few months of infection: on average 5 months (range 1–22) for natural infections and 3 months (range 1–6) for experimental infections (Moreno & Alvar, 2002). The diagnostic techniques available vary. Some, such as Western blotting, while showing excellent diagnostic performance, are not used on a large scale because of time constraints and cost. The most widely available are indirect fluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA), direct agglutination tests (DAT), and the rapid immunochromatographic assay (dipstick or strip-test).

2.1. Indirect fluorescent antibody test

The IFAT is widely used because it is easy to perform. The test is genus specific, although significant cross-reactions have been reported in individuals infected with *Trypanosoma cruzi*. For these subjects, serological tests based on specific recombinant *Leishmania* antigens would be more appropriate (see Sections B.2.2.2 and B.2.2.4 below). In Chagas' disease-free areas, the IFAT for the diagnosis of clinical VL or CanL has a sensitivity of 96% and specificity of 98%, which is similar to the ELISA. Although amastigotes from frozen sections or smears of infected organs can be used as antigen, cultured promastigotes represent the commonest antigen source.

2.1.1. Antigen preparation

- i) Harvest 3–4 ml of the liquid media of a 3-day-old culture showing flourishing promastigote growth (see Section B.1 for culture media).
- ii) Wash the organisms three times with phosphate-buffered saline (PBS), 0.16 M, pH 7.2–7.4, by centrifugation at 350 **g** for 15 minutes at room temperature.
- iii) Resuspend the final cell pellet in PBS and adjust the promastigote concentration to approximately 4×10^6 /ml with the aid of a haemocytometer.
- iv) Distribute 30 μ l of the promastigote suspension on to each circle of a multi-spot slide and allow to dry at room temperature.
- v) Fix the promastigotes in cold acetone for 10 minutes, then put the slides into a plastic box and keep in a deep freezer (-35°C) for no longer than 2–3 months.

2.1.2. Test procedure

- i) Wash the frozen antigen-coated slides in PBS and allow to dry at room temperature.
- ii) Inactivate the sera for 30 minutes in a water bath at 56°C .
- iii) Make doubling dilutions of test sera from 1/80 to 1/10,240 for human VL, and from 1/40 to 1/5120 for CanL. Positive and negative control sera, at dilutions of 1/80 and 1/160 for human VL, and of 1/40 and 1/80 for CanL, are also included in the test. No standard sera are available, but internal standards should be prepared and titrated.
- iv) Distribute 30 μ l of diluted serum samples on to each slide circle and incubate for 30 minutes at 37°C .

- v) Remove the serum samples by vigorous washing in PBS, followed by immersion of the slides in PBS for 10 minutes. Allow the slides to dry.
- vi) Distribute 30 µl of diluted fluorescein isothiocyanate (FITC)-conjugated anti-immunoglobulin on to each slide circle and incubate for 30 minutes at 37°C. FITC-conjugated anti-human and anti-dog immunoglobulins are commercially available. Follow the instructions for the appropriate dilution.
- vii) Repeat step v and mount with a cover-slip in a few drops of PBS/glycerol (50% [v/v] of each).
- viii) Read the slides under a fluorescent microscope. The highest dilution showing fluorescent promastigotes is taken to be the antibody titre.

2.1.3. Interpretation of the results

In acute human VL, the threshold titre usually ranges from 1/80 to 1/160. Asymptomatic or subclinical human disease usually results in titres below 1/80. In CanL the threshold titre ranges from 1/40 (indicative of exposure but not necessarily of established infection) to 1/160 (indicative of established infection), whereas a titre of 1/320 or above can be indicative of the disease in clinically suspected dogs (Paltrinieri *et al.*, 2010). ~~As regards other domestic mammals (e.g. cats) no standardised IFA threshold limits are available. As IFAT performance may vary in different laboratories, it is better for each laboratory to define its own threshold titre using defined positive and negative reference sera.~~ In FeL, interpretation of IFAT results is more challenging due to the absence of standardised cut-off values. However, a titre of 1/40 may be considered suspicious for exposure, while titres $\geq 1/80$ are generally regarded as positive and consistent with infection. It is important to note that antibody titres alone are insufficient for a definitive diagnosis in cats. Cats develop predominantly a Th1-type immune response mediated by IFN- γ ; however, in the presence of comorbidities (e.g. FIV, FeLV), a cytokine switch toward a Th2 profile may occur, resulting in disease progression. Positive IFAT findings should therefore be interpreted in light of clinical presentation and confirmed by PCR on appropriate samples. In seronegative or low-titre cats without evident clinical lesions, parasite detection may still be possible through cytological examination or PCR analysis of bone marrow, lymph node, or splenic samples.

2.2. Enzyme-linked immunosorbent assay

The ELISA can be carried out on serum or on a measured volume of blood. The blood is collected by needle-prick on to suitable absorbent paper strips and allowed to dry. The sample is eluted and tested at a single dilution previously determined to give an acceptable sensitivity and specificity. This test can be used for seroepidemiological surveys under field conditions.

In the classical method, the antigen is prepared as follows: promastigotes harvested from cultures are washed four times with PBS, 0.16 M, pH 7.2, at 1000 **g** for 15 minutes. The packed promastigotes are resuspended in twice their volume of distilled water, and then sonicated at medium amplitude in an ice bath. The suspension is left at 4°C overnight to allow the proteins to come into solution. After a final centrifugation at 4000 **g** for 10 minutes to eliminate the cellular debris, the overlay, representing the concentrated soluble antigen, is dispensed into vials and stored at –20°C until required. For use in the test, it is reconstituted with PBS to the predetermined optimal protein concentration (around 20 µg/ml) as measured by Lowry's method. Enzyme (usually horseradish peroxidase)-conjugated reagents consist of anti-dog goat immunoglobulins or Protein A (Hamarsheh *et al.*, 2012).

The ELISA is useful for the diagnosis of ~~Old and New World~~ leishmaniasis. Cross-reaction with *Babesia canis* and *Trypanosma* spp. has been reported (Gottstein *et al.*, 1988). According to the *Leishmania* strain used, sensitivity of the ELISA can range from 86% to 99%.

A detergent-soluble promastigote antigen has been used in ELISA instead of the crude lysate, for the diagnosis of CanL. The detergent was Triton X-100 and the proteic extract was protected with protease inhibitors. Using this method, ELISA sensitivity increased to 99.5%, while its specificity was comparable with that of the IFA test (97%) (Mancianti *et al.*, 1995).

The ELISA methods described above are based on crude antigenic preparations. A recombinant antigen from a cloned protein of *L. infantum*, called rK39, has been reported to be highly reactive to sera from human and canine visceral leishmaniasis cases when run in an ELISA format. Using 25–50 ng of the antigen, 99% specificity and sensitivity was consistently found for dogs with parasitologically proven disease (Scalone *et al.*, 2002). In HIV-positive patients, K39-ELISA showed higher sensitivity (82%) than the IFA test (54%) (Houghton *et al.*, 1998). The K39 antigen, which shows remarkable stability and reproducibility, is commercially available. More recently, a K9-K39-K26 recombinant chimeric antigen has been evaluated as a single ELISA protocol for serological diagnosis of both human and canine

Leishmania infections (Daprà *et al.*, 2008). In dogs, test specificity and sensitivity were reported to be 99.5% and 98.5%, respectively, with high concordance (K value: 0.98) with standard IFA test.

2.3. Direct agglutination test

The DAT has been described for the diagnosis of VL and CanL. After test improvement, DAT has been validated as a specific and sensitive assay for field investigations (Beelaert *et al.*, 1999; Cardoso *et al.*, 2004; Ozbek *et al.*, 2000). The antigen consists of promastigotes harvested from cultures, washed in PBS, pH 7.2, treated with 0.4% trypsin (for 45 minutes at 37°C and then washed again), and stained with 0.02% Coomassie brilliant blue. Twofold serial dilutions of serum in PBS are made in V-bottomed microtitre-plate wells; 50 µl of antigen preparation is added to each well, and the plate is then carefully shaken by hand and left for 18 hours at room temperature. The test is read visually against a white background. Positive reactions are indicated by typical light-blue aggregates, while negative samples give a clear sharp-edged blue spot.

A modified DAT for detection of specific anti-leishmanial antibodies in canine reservoir hosts is considered to be highly suitable for wide-scale epidemiological and ecological field work and diagnosis of CanL, having 100% sensitivity and 98.9% specificity (Harith *et al.*, 1988; 1989). The reliability of the test was improved by treating the test sera with 0.2 M 2-mercaptoethanol and incubating them at 37°C.

2.4. Rapid immunochromatographic assay (dipstick or strip-test)

The rapid immunochromatographic assay is easy to carry out and can be performed in veterinary clinics but has lower diagnostic sensitivity than the ELISA and IFAT: specificity is medium-high, but sensitivity is low (30–70%) (Gradoni, 2002; Mettler *et al.*, 2005; Reithinger *et al.*, 2002) and therefore it can give false negative results. When there is a strong suspicion of a false negative result using a rapid immunochromatographic assay, one of the other serology tests must be used. The value of a positive result is limited because the test does not allow antibody titre to be evaluated, however positive results can be useful for identifying individuals with parasite dissemination and for monitoring therapeutic response.

A rapid immunochromatographic assay using rK39 as antigen (K39 dipstick or strip-test, commercially available) has been evaluated in different endemic settings of VL. The nitrocellulose membrane of the test kit holds an absorbent pad at one end, a band of immobilised anti-protein A antibody (used to detect IgG) at the other (control region), and a band of rK39 antigen in the middle (test region). A protein-A-colloidal gold conjugate is used as the immunochromatographic detection reagent. One small drop (20 µl) of the serum to be examined is placed on the absorbent pad before two large drops (100 µl) of test buffer are added to the pad, and the mixture is allowed to migrate up the strip by capillary action. After 2–10 minutes, the result is positive if two distinct red lines appear (one in the test region and another in the control region), it is negative when no red line appears in the test region, and it is invalid if the control line fails to appear.

In clinical cases of human VL, two commercial brands of K39 dipstick showed 99–100% sensitivity and 95–100% specificity in India (Sundar *et al.*, 2006), 90% sensitivity and 100% specificity in Brazil (Carvalho *et al.*, 2003), and 100% sensitivity and specificity in the Mediterranean basin (Brandonisio *et al.*, 2002). In dogs with both asymptomatic and symptomatic cases of CanL, the sensitivity and the specificity of the K39 dipstick were 97% and 100%, respectively (Otranto *et al.*, 2005).

3. *Leishmania* species, subspecies or strains identification

Morphological identification enables identification of *Leishmania* at the genus level, but not at the species or subspecies level. Several techniques may be used to identify the different *Leishmania* species, subspecies or strains. Fifteen recognised *Leishmania* Identification Centres were listed by WHO in 2010.

3.1. Isoenzyme characterisation

Also known as multi locus enzyme electrophoresis (MLEE), isoenzyme characterisation is the reference method for species identification (Rioux *et al.*, 1990; WHO, 2010), although this technique requires cultivation of a large number of parasites (5×10^8 – 1×10^{10}). The principles of enzyme electrophoresis are as follows: soluble enzymes are extracted from the organisms grown in media for bulk cultivation (BHI medium, MEM/FCS/EBLB [minimal essential medium/fetal calf serum/Evans' blood lysate broth] medium, Schneider's *Drosophila* medium). A small amount of the extract is then placed in an inert supporting substance, the matrix, containing a buffer at a fixed pH. The matrix is usually starch gel, but it could equally well be absorbent cellulose acetate, acrylamide or agarose. The pH of the buffer in the matrix is usually chosen so that the isoenzymes are negatively charged. A direct current is passed through the matrix carried by the ions in the buffer. When electrophoresis is completed, most proteins

will have moved in the matrix towards the anode, depending on the amount of negative charge. If stained at this stage with a general protein stain, many bands will be seen. However, the high substrate and cofactor specificity of enzymes make it possible to stain only these proteins. Hence, the electrophoretic mobility of one particular enzyme can be compared among several organisms. The stained matrix with its collection of stained isoenzyme bands is known as a zymogram. Normally one or more extracts from reference organisms, in which the enzyme banding patterns are well documented, are included in the gel to aid the interpretation of results. Most enzymes used for characterisation purposes are stained by methods incorporating a dehydrogenase reaction. At least 12 enzymes should be examined; organisms showing identical zymograms are classified into zymodemes of a given species.

3.2. Molecular characterisation

Different molecular techniques have been developed that allow *Leishmania* characterisation at the species or strain level such as (a) PCR-restriction fragment length polymorphism (RFLP) analysis, in which the PCR products are digested by appropriate restriction enzymes and the resulting restriction fragment patterns are analysed (Marfurt *et al.*, 2003; Minodier *et al.*, 1997; Montalvo *et al.*, 2012; Volpini *et al.*, 2004); (b) multi-locus microsatellite typing (MLMT) (Kuhls *et al.*, 2011) and (c) multi-locus sequence typing (MLST) (Mauricio *et al.*, 2006). In these cases, repeated and polymorphic DNA sequences are targeted, such as ribosomal internal transcribed spacer 1 (ITS1), cysteine protease B, kinetoplast DNA minicircles, surface glycoprotein 63, heat shock protein 70, mini-exons and microsatellites (Reithinger & Dujardin, 2007; Schönian *et al.*, 2008).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

1. Vaccines Background

There are three approved patent-protected vaccines against CanL, and two vaccines initially marketed are no longer available. The first, launched in Brazil, was based on a fucose-mannose ligand enriched with glycoprotein 63 and formulated with a saponin adjuvant. The second, the first to be introduced in Europe, consisted of purified excreted/secreted antigens of *L. infantum* combined with a saponin-like adjuvant. The first patented vaccine Leish-Tec® (Ceva Santé Animale) currently on the market was developed in Brazil. Production guidelines and requirements were approved by the Brazilian Ministry of Agriculture, Livestock and Food. The vaccine contains the recombinant A2 antigen of *L. donovani* in combination with a saponin adjuvant. The second vaccine is licensed in Europe. The only currently available vaccine in Europe Letifend® (Leti Pharma laboratories), contains the active ingredient "protein Q", a recombinant protein composed of five different antigens from *L. infantum*, and does not require an additional adjuvant. The initial vaccination consists of a single injection, followed by annual booster doses. This vaccine can only be administered to healthy, seronegative dogs aged 6 months or older, confirmed through a quantitative serological test. In 2023, a new DNA vaccine was approved by the European Medicines Agency, Neoleish® (CZ Vaccines S.A.U.). It is based on the non-replicative, antibiotic resistance marker-free plasmid vector pPAL, which encodes the *L. infantum* activated protein kinase C receptor analogue (LACK). This vaccine is administered intranasally and is indicated for healthy dogs aged six months or older. None of these vaccines prevent infection; however, they can modify the course of the disease and reduce the likelihood of progression to clinically overt leishmaniasis. Beyond these licensed vaccines, several experimental formulations, including peptide-based vaccines, viral vectors and nucleic acid platforms, have been investigated in preclinical studies, with promising but as yet non-conclusive results. More recently, mRNA-based vaccines have entered early-stage research, although none has reached clinical application in dogs.

4.2. Outline of production and minimum requirements for vaccines

Leish-Tec® (Hertape Calier Saúde Animal, later Ceva, Brazil) is a recombinant vaccine composed of the *L. donovani* amastigote A2 protein and saponin as an adjuvant. It was licensed in Brazil in 2007 and remains the only approved vaccine for canine leishmaniasis (CanL) in the country. The vaccination protocol includes three subcutaneous doses at 21-day intervals for dogs aged ≥4 months, followed by annual boosters (Ghosh *et al.*, 2001). Preclinical studies in BALB/c mice demonstrated significant protection following immunisation, evidenced by reduced hepatic parasite burdens (Ghosh *et al.*, 2001). The vaccine elicited a strong antigen-specific humoral response and a mixed Th1/Th2 cellular profile. Splenocytes from vaccinated animals showed significantly elevated IFN-γ production in response to A2 stimulation, with no significant differences in IL-4 levels compared to controls (Ghosh *et al.*, 2001). In the first large-scale field trial (n>500), dogs were randomly allocated to vaccine and control groups. Infection was monitored by serology (crude antigen ELISA, IFAT), parasitological confirmation, and xenodiagnosis (subsample, n=154). Vaccine efficacy (VE) varied by endpoint: VE=71.4% (parasitological diagnosis), VE=58.1% (parasitology + xenodiagnosis), and VE=80.8% (A2 seroconversion). However, xenodiagnosis did not confirm reduced infectiousness to sand flies (Regina-Silva *et al.*, 2016).

LetiFend® (Laboratorios LETI, Spain) is a recombinant vaccine licensed in Europe in February 2016 (Cotrina *et al.*, 2018). It contains a chimeric antigen known as protein Q, composed of five fragments derived from four *L. infantum* proteins (LiP2a, LiP2b, LiP0, and histone H2A), formulated without an adjuvant. The vaccination protocol consists of a single dose followed by annual boosters and is indicated for dogs aged 6 months or older. Preclinical studies in mice demonstrated the immunogenic potential of protein Q (Molano *et al.*, 2003). When combined with live BCG and administered intraperitoneally in a three-dose schedule, it conferred protection against *L. infantum* infection in both mice and dogs (Molano *et al.*, 2003). However, due to known adverse effects associated with BCG in dogs, including local inflammation and hypersensitivity, its use as an adjuvant is discouraged (Poot *et al.*, 2009). Subsequent experiments evaluating protein Q with six alternative adjuvants failed to demonstrate protective efficacy in dogs, suggesting that BCG may have accounted for the observed protection in earlier studies (Poot *et al.*, 2009). In contrast, protein Q administered without adjuvants – corresponding to the commercial LetiFend® formulation – was shown to confer protective effects in dogs. A phase III field trial preceding vaccine licensure included 549 privately owned dogs from 19 kennels in endemic regions of France and Spain (Cotrina *et al.*, 2018). Over 2 years, dogs (275 vaccinated, 274 controls) were monitored for clinical signs, antibody response to protein Q and SLA, and parasite presence in lymphoid tissues. CanL diagnosis was based on clinical signs, positive serology, and parasite detection in bone marrow or lymph nodes. By study end, 4.7% of vaccinated dogs (n=8) and 10.2% of controls (n=19) developed confirmed CanL (p=0.048), with a calculated vaccine efficacy (VE) of 72% for preventing clinical disease. The vaccine reduced the risk of developing clinical CanL by 5-fold and confirmed CanL by 9.8-fold (Cotrina *et al.*, 2018). No adverse reactions were reported in laboratory or field evaluations (Cotrina *et al.*, 2018) and LetiFend® did not induce false-positive results in routine *L. infantum* serological tests.

Neoleish® (CZ Vaccines S.A.U.) is a DNA vaccine based on the non-replicative antibiotic resistance marker-free plasmid vector pPAL, which contains an encoding gene for the *L. infantum* activated protein kinase C receptor analogue (LACK) formulated without an adjuvant, has been approved by the European Medicines Agency in 2023. This vaccine can only be administered via intranasal, to healthy dogs of 6 months of age or older. The efficacy of the vaccine was demonstrated in a field study where dogs were naturally exposed to *L. infantum* in zones with high infection pressure over a 2-year period. In laboratory studies including experimental challenge with *Leishmania*, the vaccine reduced the severity of the disease, including clinical signs and parasites burden in bone marrow, spleen and lymph nodes. No adverse reactions were reported in laboratory or field evaluations, and in addition, the vaccine did not induce false-positive results in routine serologic tests on *L. infantum*, although efficacy data are still under study

From an immunological perspective, protective efficacy against CanL is associated with a predominantly Th1-type response characterised by IFN- γ , IL-2 and TNF- α production, which activate macrophages to control intracellular parasites. Antibody responses are useful as markers of exposure but do not reliably correlate with protection, which may explain the variability observed with some vaccines, such as Leish-Tec®, that induce mixed Th1/Th2 profiles.

The introduction of vaccination also has important implications for epidemiological surveillance. While serological monitoring may be affected by vaccine-induced antibodies, LetiFend® has the advantage of not inducing false positive results in routine tests. Although none of the available vaccines are sterilising, their widespread use can lower infection pressure in canine populations, with potential indirect benefits for human health under a One Health perspective. Longitudinal studies are still required to better define their population-level impact on transmission dynamics.

All licensed vaccines have shown a good safety profile, with adverse reactions generally mild and transient. Nonetheless, continued pharmacovigilance is essential, particularly for novel DNA-based vaccines such as Neoleish®, where field experience is still limited. Looking ahead, next-generation vaccines are being designed not only to reduce clinical disease but also to decrease infectiousness to sand flies, thereby interrupting transmission cycles. Research is also expanding towards feline leishmaniosis, as cats are increasingly recognised as potential hosts, and towards the integration of vaccination with complementary measures such as the use of repellents, vector control and management of stray dog populations.

Currently there are no available laboratory-made antigens for *Leishmania* vaccine production. There are four licensed inactivated vaccines against CanL, which are patent-protected. The first vaccine was developed in Brazil. Outlines and requirements for production were approved by the Brazilian Ministry of Agriculture, Livestock and Food Supply. The vaccine consists of the glycoprotein-enriched fraction of *L. donovani* known as 'fucose-mannose ligand' (FML). Field studies showed about 80% clinical protection conferred by the antigen administered with QuilA saponin as adjuvant, and also good immunotherapeutic efficacy when used in sick dogs (Palatnik-de-Sousa *et al.*, 2008). A second vaccine was also developed in Brazil, which uses the recombinant A2 antigen of *L. donovani* in association with a saponin adjuvant. It demonstrated 43% protection against a culture positive state in an artificial challenge model. The third vaccine is licensed in Europe. Outlines and requirements for production have been approved by the European Medicines Agency. Purified excreted-secreted proteins of *L. infantum* and with QA-21 saponin significantly reduces the risk of progressing to active infection or overt disease, with a clinical efficacy of 68%. In vaccinated dogs that developed disease and that were exposed to the bites of reared *P. perniciosus* vectors, the reduction in parasite transmission was significant when compared with matched controls. However, vaccine does not enable differentiation of vaccinated from infected animals. In addition, some safety concerns have been reported

dogs vaccinated with purified excreted-secreted proteins (Oliva *et al.*, 2014). The fourth vaccine, available in the EU, contains the active substance protein Q, which is made of different fragments of proteins from *L. infantum*. A dog vaccinated with recombinant protein Q from *L. infantum* MON-1 has five times less risk of developing clinical disease than a non-vaccinated dog. There is a lack of information concerning the interruption of *Leishmania* transmission by dogs vaccinated with recombinant protein Q from *L. infantum* MON-1 is lacking (Cotrina *et al.*, 2018). Surveillance programmes, especially those based solely on serology in dogs should be revised to take into account the impact of vaccination and to ensure epidemiological data is interpreted appropriately.

At present, a number of promising anti-leishmanial vaccines are under experimental evaluation (Coler & Reed, 2005; Das & Ali, 2012). A chimeric antigen generated from three recombinant *Leishmania* antigens screened for their ability to elicit cellular immune responses (known as Leish-111f, patent protected) and adjuvanted with monophosphoryl lipid A—stable emulsion (MPL SE), represents the first defined vaccine for leishmaniasis in humans, having completed phase 1 and 2 safety and immunogenicity testing in healthy subjects (Coler *et al.*, 2007). The same polypeptidic antigen and adjuvant failed to protect dogs from *L. infantum* infection in a phase 3 trial (Gradoni *et al.*, 2005), while conferred some protection from disease when used as an immunotherapeutic agent in dogs with mild CanL (Trigo *et al.*, 2010). Recently, a novel chimeric antigen generated from four recombinant antigens (KSAC, patent protected) has been found promising for vaccine protection in both human VL and CanL (Goto *et al.*, 2011).

2. Diagnostic biologicals (antigens for skin tests)

As the skin test is not used for diagnosis of animal infections, and the leishmanin antigen has not been internationally standardised, no recommendations can be made by WOAH.

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NB: There is a WOA Reference Laboratory for Leishmaniasis
(please consult the WOA Web site:

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

Please contact the WOA Reference Laboratory for any further information on
diagnostic tests, reagents and vaccines for leishmaniasis

NB: FIRST ADOPTED IN 1991 AS LEISHMANIASIS. MOST RECENT UPDATES ADOPTED IN 2021.

Appendix 1

Canine leishmaniosis Intended purpose of test: population freedom from infection

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Cytological examination</u> ☐ ☐ ☐ <u>Dog</u>	<u>Aspirates (lymph node, bone marrow, spleen); target: <i>Leishmania</i> amastigotes</u>	<u>Low overall sensitivity in asymptomatic or early infections; not appropriate for large-scale testing; BLCM not applicable</u>	<u>Typically, clinical suspects only; not representative of general population or healthy animals</u>	<u>Diagnostic utility confirmed for clinical cases only; no validation data for surveillance or certification purposes</u>	<u>Useful in clinical settings; allows direct observation of parasite</u>	<u>Invasive, low throughput, poor sensitivity in asymptomatic carriers; not feasible for population-level application</u>	<u>Solano-Gallego <i>et al.</i> (2009)</u>
<u>Histological examination</u> ☐ ☐ ☐ <u>Dog</u>	<u>Tissue biopsies (skin, lymph node, spleen); target: <i>Leishmania</i> amastigotes in macrophages</u>	<u>Moderate sensitivity, especially in early or asymptomatic infections; specificity high when interpreted by experienced pathologists; no BLCM used</u>	<u>Primarily symptomatic dogs with visible lesions; not suitable for screening general population</u>	<u>Diagnostic value confirmed for clinical cases; not validated for population-level surveillance</u>	<u>Provides histopathological context; may detect parasite in inflamed tissues</u>	<u>Invasive, low throughput, requires biopsy and pathology services; not feasible or sensitive for detecting freedom from infection in populations</u>	<u>Solano-Gallego <i>et al.</i> (2009)</u>
<u>Isolation in culture</u> ☐ ☐ ☐ <u>Dog</u>	<u>Aspirates from spleen, bone marrow, or lymph nodes; target: viable <i>Leishmania</i> parasites cultured in NNN, Schneider's, or EMTM media</u>	<u>High specificity (~100%) but variable sensitivity (60–90%) depending on sample site and parasite burden; no BLCM used</u>	<u>Validated primarily in symptomatic dogs; no evidence of suitability for detecting subclinical infections in healthy populations</u>	<u>Documented in clinical research; not validated for surveillance or population-level certification</u>	<u>Confirms presence of viable parasite; allows downstream molecular or drug resistance testing</u>	<u>Requires invasive sampling, specific culture conditions, and weeks for growth; not suitable for large-scale or asymptomatic screening</u>	<u>Solano-Gallego <i>et al.</i> (2009); Gradoni (2002)</u>
<u>Molecular methods – PCR</u> ☒ ☒ ☐ <u>Dog</u>	<u>Blood, bone marrow, lymph node aspirates; target: <i>Leishmania</i> DNA (kDNA, SSU rRNA, ITS1, etc.)</u>	<u>Sensitivity: 85–100%; Specificity: >95%; validated against culture and serology; BLCM rarely used</u>	<u>Dogs from endemic and non-endemic areas; includes subclinical infections, though intermittent parasitaemia may affect results</u>	<u>Described in peer-reviewed studies (e.g. Maia & Campino, 2008); not all protocols WOAH-validated</u>	<u>High specificity; can detect asymptomatic infections; suitable for confirmation</u>	<u>Costly and technically demanding; sensitivity may be affected by sampling time and type; false negatives possible in chronic low-parasitaemia infections</u>	<u>Maia & Campino (2008); Solano-Gallego <i>et al.</i> (2017)</u>
<u>IFAT</u> ☒ ☒ ☒ <u>Dog</u>	<u>Serum; target: anti-<i>Leishmania infantum</i> IgG antibodies</u>	<u>Sensitivity: 80–95%; Specificity: 85–98%; depends on titre cut-off and antigen quality; BLCM used in</u>	<u>Dogs from endemic and non-endemic areas, including subclinical cases; representative of general population</u>	<u>Validated and widely used in reference labs; included in WOAH Manual; Solano-Gallego <i>et al.</i>, 2009</u>	<u>Standardised, accepted for certification purposes; suitable for screening and surveillance</u>	<u>Requires trained personnel and fluorescence microscopy; possible cross-reactivity; lower sensitivity in early infection</u>	<u>Solano-Gallego <i>et al.</i> (2009); Moreno & Alvar (2002)</u>

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
		<u>some multicentre validations</u>					
<u>ELISA</u> ☒ ☒ ☒ <u>Dog</u>	<u>Serum or plasma; target: anti-<i>Leishmania infantum</i> antibodies (IgG) against crude or recombinant antigens</u>	<u>Sensitivity: 85–98%; Specificity: 90–99%; validated against IFAT and PCR; some kits evaluated using ROC analysis and BLCM</u>	<u>Dogs from various epidemiological contexts; suitable for detecting both clinical and subclinical infections</u>	<u>Extensively evaluated in scientific literature; several commercial kits in use; partial WOA validation</u>	<u>High-throughput, standardised protocols; suitable for mass screening and certification programmes</u>	<u>Performance depends on antigen source; false negatives in very early infection; inter-lab variability if not standardised</u>	<u>Solano-Gallego <i>et al.</i> (2009); Reithinger & Dujardin (2007)</u>
<u>DAT</u> ☒ ☐ ☐ <u>Dog</u>	<u>Serum; target: anti-<i>Leishmania infantum</i> IgG antibodies using fixed promastigote antigen</u>	<u>Sensitivity: 80–95%; Specificity: 85–98%; depends on antigen quality and titre cut-off; some studies use ROC analysis; BLCM rarely applied</u>	<u>Mostly symptomatic or subclinical dogs; limited data on healthy or pre-movement certified populations</u>	<u>Validated for human VL; adapted for CanL in epidemiological studies; no formal WOA validation for population freedom</u>	<u>Technically simple; cost-effective; useful in field surveys and low-resource settings</u>	<u>Requires overnight incubation; subjective interpretation; not sufficiently standardised across labs for certification use; limited validation in asymptomatic populations</u>	<u>Solano-Gallego <i>et al.</i> (2009); Babakhan <i>et al.</i> (2009)</u>
<u>Rapid immune-chromatographic assay</u> ☐ ☐ ☐ <u>Dog</u>	<u>Whole blood, serum, or plasma; target: anti-<i>Leishmania infantum</i> antibodies (typically rK39 recombinant antigen)</u>	<u>Sensitivity: 70–90%; Specificity: 85–98%; depending on the brand; limited comparative studies for low-prevalence populations; BLCM not used</u>	<u>Evaluated mainly in clinical and field settings; limited validation in healthy, asymptomatic animals or in low-prevalence areas</u>	<u>Used in veterinary clinics for individual diagnosis; not validated for mass screening or freedom-from-disease certification</u>	<u>Rapid, user-friendly, minimal equipment; suitable for preliminary diagnosis in field settings</u>	<u>Not quantitative; may miss early or low-titre infections; high inter-kit variability; not suitable for demonstrating absence of infection in populations</u>	<u>Palatnik-de-Sousa <i>et al.</i> (2008); Solano-Gallego <i>et al.</i> (2009);</u>

Appendix 2

Canine leishmaniosis

Intended purpose of test: individual animal freedom from infection prior to movement

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Cytological examination</u> ☐ ☐ ☐ <u>Dog</u>	<u>Aspirates (lymph node, spleen, bone marrow); target: <i>Leishmania</i> amastigotes</u>	<u>Low sensitivity in asymptomatic stages; no data for healthy animals in certification programmes; no BLCM used</u>	<u>Symptomatic dogs; not representative of healthy or subclinically infected animals</u>	<u>No validation for certification use; clinical diagnostic tool only</u>	<u>Direct parasite visualisation in high-load cases</u>	<u>Invasive, insensitive in early infections; not suitable for pre-movement certification</u>	<u>Solano-Gallego <i>et al.</i> (2009); Baneth <i>et al.</i> (2008)</u>
<u>Histological examination</u> ☐ ☐ ☐ <u>Dog</u>	<u>Tissue biopsies (skin, lymph node, spleen); target: <i>Leishmania</i> amastigotes in macrophages</u>	<u>Moderate sensitivity in symptomatic cases; unsuitable for detecting low-level infections; no BLCM</u>	<u>Symptomatic dogs; not designed for asymptomatic screening</u>	<u>Diagnostic value limited to confirmed clinical cases</u>	<u>Contextual tissue analysis; confirms presence of parasite in lesions</u>	<u>Invasive, low throughput, not validated for certification or screening healthy animals</u>	<u>Solano-Gallego <i>et al.</i> (2009)</u>
<u>Isolation in culture</u> ☒ ☐ ☐ <u>Dog</u>	<u>Aspirates (bone marrow, spleen); target: viable <i>Leishmania</i> parasites (NNN, Schneider's, EMTM)</u>	<u>High specificity (~100%); variable sensitivity depending on parasite burden; no BLCM</u>	<u>Clinical cases; limited data on asymptomatic dogs</u>	<u>Validated in clinical settings; not standardised for movement certification</u>	<u>Confirms viable parasite; useful for drug sensitivity and strain typing</u>	<u>Long culture time; low yield in low-parasitaemia cases; invasive and impractical for routine certification</u>	<u>Solano-Gallego <i>et al.</i> (2009); Gradoni (2002)</u>
<u>Molecular methods – PCR</u> ☒ ☒ ☒ <u>Dog</u>	<u>Blood, bone marrow, lymph node aspirates; target: <i>Leishmania</i> DNA (kDNA, SSU rRNA, ITS1)</u>	<u>Sensitivity: 85–100%; Specificity: >95%; high accuracy in detecting low-level infections; no BLCM</u>	<u>Validated in both clinical and subclinical dogs; suitable for healthy animals</u>	<u>Extensively evaluated in peer-reviewed studies (Maia & Campino, 2008); not fully WOA-validated</u>	<u>High sensitivity and specificity; effective in asymptomatic animals; suitable for certification</u>	<u>Requires well-equipped lab; risk of false negatives in chronic or latent infections; costly</u>	<u>Solano-Gallego <i>et al.</i> (2009); Moreno & Alvar (2002)</u>
<u>IFAT</u> ☒ ☒ ☐ <u>Dog</u>	<u>Serum; anti-<i>Leishmania infantum</i> IgG</u>	<u>Sensitivity: 80–95%; Specificity: 85–98%; depends on antigen, cut-off, and technique</u>	<u>Validated in clinical and subclinical dogs; used for screening and certification</u>	<u>Widely used in reference labs; included in WOA- Manual</u>	<u>Standardised method; accepted in trade/export protocols; suitable for large-scale screening</u>	<u>Requires fluorescence microscope and trained personnel; possible cross-reactions</u>	<u>Solano-Gallego <i>et al.</i> (2009); Moreno & Alvar (2002)</u>
<u>ELISA</u> ☒ ☒ ☐ <u>Dog</u>	<u>Serum/plasma; anti-<i>Leishmania</i> IgG antibodies (crude or recombinant antigen)</u>	<u>Sensitivity: 85–98%; Specificity: 90–99%; validated against IFAT and PCR</u>	<u>Includes asymptomatic and clinical dogs; suitable for certification screening</u>	<u>Extensively validated in literature; some kits partially WOA-listed</u>	<u>High throughput; automated versions available; consistent performance with recombinant antigens</u>	<u>May miss early infection; inter-lab variability if not standardised</u>	<u>Solano-Gallego <i>et al.</i> (2009); Reithinger & Dujardin (2007)</u>

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
DAT ☒ ☐ ☐ Dog	Serum; target: anti- <i>Leishmania infantum</i> IgG antibodies using fixed whole promastigotes	Sensitivity: 80–95%; Specificity: 85–98%; results vary by batch and technique; limited ROC data; BLCM not commonly applied	Mostly symptomatic and subclinical dogs; limited evidence for healthy pre-movement populations	Adapted from human VL applications; used in field studies; not officially validated for canine movement certification	Technically simple; does not require sophisticated equipment; useful in resource-limited contexts	Time-consuming (overnight incubation); subjective reading; limited standardisation and external quality control; not widely accepted for international certification	Babakhan <i>et al.</i> (2009); Solano-Gallego <i>et al.</i> (2009)
Rapid immune-chromatographic assay ☐ ☐ ☐ Dog	Whole blood, serum, or plasma; target: anti- <i>Leishmania infantum</i> antibodies (commonly rK39 antigen)	Sensitivity: 70–90%; Specificity: 85–98%; performance varies significantly between commercial brands; limited ROC/validation data; no BLCM	Validated primarily in symptomatic dogs in clinical settings; lacks data for asymptomatic or healthy populations being moved	Not validated for pre-movement certification or international trade; clinical-use only	Rapid and user-friendly; can be used in field conditions without specialised equipment	Limited sensitivity in early/subclinical infections; qualitative only; not accepted for certification purposes; significant inter-batch and inter-kit variability	Palatnik-de-Sousa <i>et al.</i> (2008); Solano-Gallego <i>et al.</i> (2009);

Appendix 3

Canine leishmaniosis

Intended purpose of test: contribute to eradication policies

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Cytological examination</u> ☐ ☐ ☐ <u>Dog</u>	<u>Aspirates from spleen, bone marrow, or lymph nodes; target: <i>Leishmania</i> amastigotes</u>	<u>Sensitivity: variable (30–70%); specificity >90% in clinical cases; poor detection in subclinical or early infections; no BLCM applied</u>	<u>Used primarily in symptomatic dogs; not designed for screening or monitoring populations under eradication programmes</u>	<u>Only validated for confirmation in individual clinical cases; no support for use in large-scale or longitudinal surveillance</u>	<u>Inexpensive, rapid, and useful in clinical diagnosis</u>	<u>Low throughput, invasive, requires expertise in microscopy; low sensitivity in asymptomatic dogs; not scalable for eradication strategies</u>	<u>Solano-Gallego <i>et al.</i> (2009); Baneth <i>et al.</i> (2008)</u>
<u>Histological examination</u> ☐ ☐ ☐ <u>Dog</u>	<u>Tissue biopsies (e.g. skin, lymph node, spleen); target: <i>Leishmania</i> amastigotes within host cells</u>	<u>Moderate sensitivity in symptomatic animals (~50–80%); high specificity when interpreted by trained pathologists; not validated for subclinical infections; no BLCM</u>	<u>Clinical cases only; not suitable for detecting or monitoring infection in apparently healthy animals or populations</u>	<u>Used as a confirmatory tool in individual diagnostics; not validated for population-level eradication programmes</u>	<u>Provides detailed histopathological context; may detect parasite in lesion sites</u>	<u>Requires biopsy and histological processing; low throughput; invasive and costly; not scalable or sensitive enough for eradication efforts</u>	<u>Solano-Gallego <i>et al.</i> (2009); Baneth <i>et al.</i> (2008)</u>
<u>Isolation in culture</u> ☐ ☐ ☐ <u>Dog</u>	<u>Aspirates from spleen, bone marrow, or lymph nodes; target: viable <i>Leishmania</i> parasites cultured in NNN, Schneider's, or EMTM media</u>	<u>Sensitivity: 60–90% depending on sample quality; specificity: ~100%; performance declines in asymptomatic or chronic cases; no BLCM used</u>	<u>Validated in symptomatic dogs; not suited for detecting low-level infections common in reservoir animals or early infection</u>	<u>Validated in clinical settings; not designed for large-scale application in eradication program</u>	<u>Confirms presence of viable parasite; useful for research and parasite characterisation</u>	<u>Invasive, laborious, requires weeks for culture, low yield in subclinical infections; impractical for large-scale or repeated sampling in eradication programmes</u>	<u>Solano-Gallego <i>et al.</i> (2009); Gradoni (2002)</u>
<u>Molecular methods – PCR</u> ☒ ☐ ☐ <u>Dog</u>	<u>Blood, lymph node aspirates, bone marrow, skin; target: <i>Leishmania</i> DNA (e.g. kinetoplast DNA, ITS1, SSU rRNA)</u>	<u>Sensitivity: 85–100%; Specificity: >95%; however, intermittent parasitaemia and sample quality affect reliability for eradication targets; BLCM not routinely used</u>	<u>Dogs from both clinical and subclinical contexts; suitable for detection but not practical for repeated population-wide testing</u>	<u>Extensively used in research and diagnostics; partially standardised; not fully WOAH-validated for eradication program use</u>	<u>High analytical sensitivity and specificity; allows early detection of infection; useful in targeted surveillance or reservoir host studies</u>	<u>Requires well-equipped lab and skilled personnel; costly and labour-intensive for wide-scale use; sensitivity varies by sample and stage of infection</u>	<u>Maia & Campino (2008); Solano-Gallego <i>et al.</i> (2017)</u>
<u>IFAT</u> ☒ ☒ ☒ <u>Dog</u>	<u>Serum; target: anti-<i>Leishmania infantum</i> IgG antibodies</u>	<u>Sensitivity: 80–95%; Specificity: 85–98%, depending on antigen, cut-off titre, and lab conditions; ROC and</u>	<u>Broadly validated in endemic and non-endemic areas; includes both symptomatic and asymptomatic animals;</u>	<u>Recognised by WOA; widely used in reference laboratories; performance supported</u>	<u>Reliable, standardised, suitable for mass screening and monitoring programmes;</u>	<u>Requires fluorescence microscopy and trained personnel; cross-reactivity possible with other trypanosomatids; needs</u>	<u>Solano-Gallego <i>et al.</i> (2009); Moreno & Alvar (2002)</u>

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
		<u>BLCM used in some comparative studies</u>	<u>suitable for eradication monitoring</u>	<u>by large-scale seroprevalence studies</u>	<u>acceptable for international trade and certification</u>	<u>standardisation of cut-off values</u>	
<u>ELISA</u> ☒ ☒ ☒ <u>Dog</u>	<u>Serum or plasma; target: anti-<i>Leishmania infantum</i> IgG antibodies (crude or recombinant antigens)</u>	<u>Sensitivity: 85–98%; Specificity: 90–99%; performance confirmed via ROC analyses and in large-scale screening; some BLCM applications in multi-centre studies</u>	<u>Validated on both symptomatic and asymptomatic dogs; large epidemiological datasets confirm its use for control and eradication strategies</u>	<u>Commonly used in national surveillance and eradication campaigns; some commercial kits partially WOAH-validated</u>	<u>High-throughput, cost-effective, automatable; consistent performance; reliable for monitoring trends and identifying reservoirs</u>	<u>Cut-off interpretation may vary by kit; recombinant antigens improve specificity but may miss some field strains; limited in early infection</u>	<u>Solano-Gallego <i>et al.</i> (2009); Reithinger & Dujardin (2007); Chapter 3.1.20 Rift Valley fever?</u>
<u>DAT</u> ☐ ☐ ☐ <u>Dog</u>	<u>Serum; target: anti-<i>Leishmania infantum</i> IgG antibodies using fixed promastigote antigen</u>	<u>Sensitivity: 80–95%; Specificity: 85–98%; results vary by antigen batch; limited inter-laboratory standardisation; ROC used in some studies</u>	<u>Applied mainly in clinical or field screening; not validated for continuous monitoring in structured eradication programmes</u>	<u>Used in epidemiological studies and human VL diagnosis; not widely accepted or validated for veterinary eradication campaigns</u>	<u>Inexpensive, field applicable, technically simple</u>	<u>Long incubation time (overnight), subjective interpretation, batch-to-batch variability, and low reproducibility across labs limit its utility in standardised eradication programmes</u>	<u>Babakhan <i>et al.</i> (2009); Solano-Gallego <i>et al.</i> (2009); Chapter 3.1.20 Rift Valley fever?</u>
<u>Rapid immunochromatographic assay</u> ☐ ☐ ☐ <u>Dog</u>	<u>Whole blood, serum, or plasma; target: anti-<i>Leishmania infantum</i> antibodies (typically rK39 antigen)</u>	<u>Sensitivity: 70–90%; Specificity: 85–98%; considerable variability between commercial kits; lacks robust validation for surveillance or eradication use</u>	<u>Evaluated primarily in symptomatic or field-diagnosed dogs; not suited for identifying asymptomatic carriers or for longitudinal monitoring</u>	<u>Not validated for structured eradication campaigns; lacks standardisation and regulatory acceptance for this purpose</u>	<u>Easy to use; rapid results; minimal equipment needed</u>	<u>Poor sensitivity in subclinical or early infections; qualitative only; high variability between kits and lots; not adequate for supporting official control programmes</u>	<u>Palatnik-de-Sousa <i>et al.</i> (2008); Solano-Gallego <i>et al.</i> (2009);</u>

Appendix 4

Canine leishmaniosis

Intended purpose of test: confirmation of clinical cases

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Cytological examination</u> ☒ ☒ ☒ <u>Dog</u>	<u>Aspirates from lymph node, spleen, or bone marrow; target: <i>Leishmania</i> amastigotes</u>	<u>Sensitivity: 30–70%, depending on sample site and parasitic load; Specificity: >90% when compared to PCR or culture; BLCM not applied</u>	<u>Symptomatic dogs with clinical signs and compatible lesions; routinely applied in veterinary diagnostics</u>	<u>Well-established in veterinary parasitology and internal medicine; standard procedure in endemic regions</u>	<u>Simple, fast, and cost-effective; allows direct visual identification of the parasite; highly specific</u>	<u>Sensitivity depends on operator skill, sample site and parasite burden; may yield false negatives in early or low-grade infections</u>	<u>Solano-Gallego <i>et al.</i> (2009); Baneth <i>et al.</i> (2008); Chapter 3.1.20 Rift Valley fever?</u>
<u>Histological examination</u> ☒ ☒ ☐ <u>Dog</u>	<u>Tissue biopsies (e.g. skin, lymph node, spleen); target: <i>Leishmania</i> amastigotes in macrophages within inflammatory lesions</u>	<u>Sensitivity: ~50–80% in symptomatic dogs; Specificity: >90% with expert interpretation; limited ROC studies; BLCM not used</u>	<u>Symptomatic dogs with compatible clinical lesions; not applicable to asymptomatic or screening contexts</u>	<u>Described in veterinary pathology literature; standard procedure in diagnostic histopathology; not WOAH-validated</u>	<u>Confirms presence of parasite in tissue context; supports differential diagnosis and pathogenesis in low-study</u>	<u>Invasive; requires histopathological facilities and skilled personnel; less sensitive than PCR in low-parasite-load tissues</u>	<u>Solano-Gallego <i>et al.</i> (2009); Baneth <i>et al.</i> (2008)</u>
<u>Isolation in culture</u> ☒ ☒ ☐ <u>Dog</u>	<u>Aspirates from spleen, bone marrow, or lymph nodes; target: viable <i>Leishmania</i> parasites cultured in NNN, Schneider's, or EMTM media</u>	<u>Sensitivity: 60–90%, depending on sample site, stage, and parasite load; Specificity: ~100%; no BLCM used</u>	<u>Symptomatic dogs with high parasite burden; particularly effective in advanced clinical cases</u>	<u>Validated and used in clinical and research settings; standard method for definitive diagnosis and parasite characterisation</u>	<u>Confirms presence of live parasite; enables further typing, drug susceptibility testing, and molecular epidemiology</u>	<u>Requires invasive sampling and sterile technique; time-consuming (may take 1–4 weeks); low sensitivity in low-parasite-load or treated animals</u>	<u>Solano-Gallego <i>et al.</i> (2009); Gradoni (2002)</u>
<u>Molecular methods – PCR</u> ☒ ☒ ☒ <u>Dog</u>	<u>Blood, lymph node aspirates, bone marrow, skin biopsies; target: <i>Leishmania</i> DNA (e.g. kDNA minicircle, SSU rRNA, ITS1)</u>	<u>Sensitivity: 85–100%; Specificity: >95%; validated in comparison to parasitology and serology; BLCM used in some studies</u>	<u>Symptomatic and subclinical dogs; highly representative of clinical spectrum; suitable for both high and low parasite burdens</u>	<u>Extensively validated in research and clinical diagnostics; many protocols standardised in reference laboratories</u>	<u>High sensitivity and specificity; detects parasite DNA in early or atypical infections; non-invasive sampling possible; allows species identification</u>	<u>Requires molecular lab and technical expertise; risk of contamination; false negatives possible in chronic low-parasitaemia cases</u>	<u>Maia & Campino (2008); Solano-Gallego <i>et al.</i> (2017); Chapter 3.1.20 Rift Valley fever?</u>
<u>IFAT</u> ☒ ☒ ☐ <u>Dog</u>	<u>Serum; target: anti-<i>Leishmania infantum</i> IgG antibodies</u>	<u>Sensitivity: 80–95%; Specificity: 85–98%; commonly compared with ELISA and PCR;</u>	<u>Validated in both symptomatic and subclinical dogs; widely accepted as a reference test for CanL</u>	<u>Standardised in reference laboratories and included in WOAH <i>Terrestrial Manual</i></u>	<u>Reliable, standardised method for diagnosis; quantitative titres allow</u>	<u>Requires fluorescence microscope and trained personnel; cross-reactivity possible with other</u>	<u>Solano-Gallego <i>et al.</i> (2009); Moreno & Alvar (2002)</u>

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
		ROC and BLCM used in some validations			disease staging and follow-up	trypanosomatids; not fully reliable in early infection	
<u>ELISA</u> ☒ ☒ ☐ <u>Dog</u>	<u>Serum or plasma; target: anti-<i>Leishmania infantum</i> IgG antibodies (using crude or recombinant antigen)</u>	<u>Sensitivity: 85–98%; Specificity: 90–99%; validated against parasitological and molecular tests; ROC analyses commonly used; some use of BLCM</u>	<u>Symptomatic and subclinical dogs; used extensively in both clinical and epidemiological studies</u>	<u>Several commercial ELISA kits validated; some included in official programmes or recommended by reference laboratories</u>	<u>High-throughput, quantitative, automatable; good diagnostic accuracy; titres can support clinical staging and follow-up</u>	<u>May not distinguish active from past infection; performance varies with antigen type and manufacturer; interpretation can require confirmation with PCR or parasitology</u>	<u>Solano-Gallego <i>et al.</i> (2009); Reithinger & Dujardin (2007); Chapter 3.1.20 Rift Valley fever?</u>
<u>DAT</u> ☒ ☒ ☐ <u>Dog</u>	<u>Serum; target: anti-<i>Leishmania infantum</i> IgG antibodies using fixed promastigote antigen</u>	<u>Sensitivity: 80–95%; Specificity: 85–98%; some studies with ROC analysis; limited use of BLCM</u>	<u>Symptomatic and subclinically infected dogs; used in field settings and epidemiological investigations</u>	<u>Originally validated in human VL; adapted to canine use in several studies; not WOAH-listed for CanL</u>	<u>Technically simple; no sophisticated equipment needed; useful for screening in resource-limited settings</u>	<u>Requires long incubation (overnight); subjectivity in interpreting agglutination; lower standardisation than ELISA/IFAT</u>	<u>Babakhan <i>et al.</i> (2009); Solano-Gallego <i>et al.</i> (2009); Chapter 3.1.20 Rift Valley fever?</u>
<u>Rapid Immuno-chromatographic assay</u> ☒ ☒ ☐ <u>Dog</u>	<u>Whole blood, serum, or plasma; target: anti-<i>Leishmania infantum</i> antibodies (commonly rK39 antigen)</u>	<u>Sensitivity: 70–90%; Specificity: 85–98%; varies by manufacturer and antigen used; limited use of BLCM</u>	<u>Dogs with clinical signs; used in clinics and field settings; performance lower in subclinical cases</u>	<u>Commercially available kits; evaluated in clinical diagnosis, but not standardised across labs</u>	<u>Fast (results in 10–15 minutes); simple to use; can be performed in field conditions or general practice</u>	<u>Limited sensitivity in low-titre or early infections; qualitative only; significant variability across kits; confirmation with other tests often required</u>	<u>Palatnik-de-Sousa <i>et al.</i> (2008); Solano-Gallego <i>et al.</i> (2009)</u>

Appendix 5

Canine leishmaniosis

Intended purpose of test: prevalence of infection -- surveillance

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Cytological examination</u> ☐ ☐ ☐ <u>Dog</u>	<u>Aspirates from spleen, lymph node, or bone marrow; target: <i>Leishmania</i> amastigotes</u>	<u>Sensitivity: 30–70% depending on parasite burden and sampling site; Specificity: >90%; not suitable for detecting low-level infections in asymptomatic animals</u>	<u>Primarily symptomatic animals; not representative of general dog populations or carriers in endemic areas</u>	<u>Validated as a confirmatory clinical tool; not suitable for population-based studies or large-scale application</u>	<u>Rapid and specific in high-parasite-load samples; useful in individual diagnosis</u>	<u>Invasive; requires skilled operators; poor sensitivity in asymptomatic infections; unsuitable for epidemiological surveys</u>	<u>Solano-Gallego <i>et al.</i> (2009); Baneth <i>et al.</i> (2008)</u>
<u>Histological examination</u> ☐ ☐ ☐ <u>Dog</u>	<u>Tissue biopsies (e.g. skin, lymph node, spleen); target: <i>Leishmania</i> amastigotes in inflammatory lesions</u>	<u>Sensitivity: ~50–80% in symptomatic dogs; Specificity: >90%; very low sensitivity in asymptomatic cases; BLCM not used</u>	<u>Designed for dogs with clinical lesions; unsuitable for healthy populations in surveillance studies</u>	<u>Standard diagnostic method in pathology; not validated or practical for epidemiological prevalence surveys</u>	<u>Valuable for individual case diagnosis; confirms parasite within tissue context</u>	<u>Invasive; low-throughput; requires histopathology lab; poor sensitivity in asymptomatic infections; not feasible for large-scale or repeated sampling</u>	<u>Solano-Gallego <i>et al.</i> (2009); Baneth <i>et al.</i> (2008)</u>
<u>Isolation in culture</u> ☐ ☐ ☐ <u>Dog</u>	<u>Aspirates from spleen, bone marrow, or lymph nodes; target: viable <i>Leishmania</i> promastigotes grown in NNN, Schneider's, or EMTM media</u>	<u>Sensitivity: 60–90% in clinical cases; much lower in asymptomatic or low-parasitaemia animals; Specificity: ~100%; no BLCM applied</u>	<u>Primarily symptomatic dogs; not representative of general or subclinical populations required for surveillance</u>	<u>Validated as a definitive diagnostic method; not suitable for screening large populations</u>	<u>Confirms active infection with viable parasite; enables further molecular or phenotypic studies</u>	<u>Laborious, time-consuming (1–4 weeks), invasive sampling required, low sensitivity in asymptomatic carriers, impractical for large-scale surveys</u>	<u>Solano-Gallego <i>et al.</i> (2009); Gradoni (2002)</u>
<u>Molecular methods – PCR</u> ☒ ☒ ☐ <u>Dog</u>	<u>Blood, bone marrow, lymph node aspirates, skin biopsies; target: <i>Leishmania</i> DNA (e.g. kDNA minicircle, SSU rRNA, ITS1)</u>	<u>Sensitivity: 85–100% in symptomatic dogs, lower in asymptomatic carriers due to intermittent parasitaemia; Specificity: >95%; validated with parasitology/serology; BLCM rarely used</u>	<u>Symptomatic and asymptomatic dogs; appropriate for targeted surveillance in endemic areas, but limited scalability</u>	<u>Widely studied; used in focused epidemiological research; not broadly validated for population-level surveillance</u>	<u>High specificity and sensitivity; detects active infection even in absence of antibody response; useful in sentinel surveillance or targeted risk areas</u>	<u>Costly, requires advanced lab infrastructure and skilled personnel; sensitivity affected by sampling site and infection stage; not ideal for large-scale field surveys</u>	<u>Maia & Campino (2008); Solano-Gallego <i>et al.</i> (2017)</u>

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
IFAT ☒ ☒ ☒ Dog	Serum; target: anti- <i>Leishmania infantum</i> IgG antibodies	Sensitivity: 80–95%; Specificity: 85–98%; used as reference serological test in many validation studies; BLCM applied in some population-level comparisons	Symptomatic and asymptomatic dogs in endemic and non-endemic regions; validated for cross-sectional surveys	Standardised and recommended by WOA; broadly used in national and international surveillance programmes	Highly reliable; quantifiable; suitable for large-scale screening and longitudinal seroprevalence studies	Requires trained personnel and fluorescence microscopy; inter-laboratory variability if cut-offs are not harmonised; possible cross-reactivity	Solano-Gallego <i>et al.</i> (2009); Moreno & Alvar (2002)
ELISA ☒ ☒ ☒ Dog	Serum or plasma; target: anti- <i>Leishmania infantum</i> IgG antibodies (using crude or recombinant antigens)	Sensitivity: 85–98%; Specificity: 90–99%; validated with parasitological and molecular tests; ROC commonly used; some BLCM applications	Symptomatic and asymptomatic dogs from both endemic and non-endemic areas; widely validated in population studies	Commercially available kits evaluated in multicentric surveillance programmes; some with WOA recognition	High-throughput; cost-effective; suitable for cross-sectional and longitudinal surveys; allows automation and large-scale use	Requires quality control and standardisation of cut-offs; false positives possible due to exposure without active infection; some inter-kit variability	Solano-Gallego <i>et al.</i> (2009)
DAT ☒ ☒ ☐ Dog	Serum; target: anti- <i>Leishmania infantum</i> IgG antibodies using fixed promastigote antigen	Sensitivity: 80–95%; Specificity: 85–98%; validated against IFAT and ELISA in several field studies; some ROC analyses	Dogs in endemic and non-endemic areas; used in field epidemiology and seroprevalence studies, including asymptomatic animals	Extensively used in human VL surveillance; adapted for CanL; not standardised across all veterinary laboratories	Simple, low-cost, and effective for use in field or resource-limited settings; minimal equipment required	Requires overnight incubation and careful visual interpretation; limited availability in some regions; batch variability can affect reproducibility	Babakhan <i>et al.</i> (2009); Solano-Gallego <i>et al.</i> (2009)
Rapid immuno-chromatographic assay ☐ ☐ ☐ Dog	Whole blood, serum, or plasma; target: anti- <i>Leishmania infantum</i> antibodies (typically rK39 antigen)	Sensitivity: 70–90%; Specificity: 85–98%; significant variability across commercial kits; poor performance in early/subclinical infections	Mainly symptomatic dogs in clinical or field diagnosis; underperforms in detecting infection in asymptomatic carriers	Not validated for population-level surveillance; lacks standardisation and reproducibility across different epidemiological settings	Fast and easy to use in the field or in clinics; requires no equipment	Qualitative only; poor sensitivity in early/asymptomatic infection; inconsistent results across brands; not suitable for accurate prevalence estimates	Palatnik-de-Sousa <i>et al.</i> (2008); Solano-Gallego <i>et al.</i> (2009);

Appendix 6

Feline leishmaniosis

Intended purpose of test: contribute to eradication policies; confirmation of clinical cases; prevalence of infection – surveillance; immune status in individual animals or populations post-vaccination

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Cytological examination</u> ☒ ☒ ☐ <u>for confirmation of clinical cases</u> <u>Cat</u>	<u>Fine needle aspirates (FNA) of lymph nodes, skin nodules, spleen, bone marrow; target: Leishmania amastigotes</u>	<u>Sensitivity: moderate (~40–70%) depending on tissue and parasitemia; Specificity: high when amastigotes are observed; no BLCM used in cats</u>	<u>Symptomatic cats with nodular, ulcerative, or mucocutaneous lesions; confirmed mostly in Mediterranean and endemic regions</u>	<u>No formal WOA validation; widely used in documented clinical cases in veterinary literature</u>	<u>Quick and specific when positive; minimally invasive; useful in cutaneous and nodular forms</u>	<u>Low sensitivity in early/systemic or non-cutaneous disease; parasite may be sparse; highly operator-dependent</u>	<u>Pennisi <i>et al.</i> (2015); Solano-Gallego <i>et al.</i> (2009); Brianti <i>et al.</i> (2020)</u>
<u>Histological examination</u> ☒ ☒ ☐ <u>for confirmation of clinical cases</u> <u>Cat</u>	<u>Tissue biopsies (skin, mucosa, lymph nodes, spleen); target: Leishmania amastigotes in inflammatory lesions</u>	<u>Sensitivity: moderate (~50–75%) depending on tissue and parasite load; Specificity: high with experienced histopathologist; no BLCM used</u>	<u>Symptomatic cats with dermal, mucocutaneous, or systemic lesions; mostly from endemic regions</u>	<u>Not formally validated by WOA; described in several clinical and pathological case series</u>	<u>Confirms parasite within tissue architecture; supports differential diagnosis; may detect concurrent pathology</u>	<u>Invasive; requires biopsy and histopathological processing; less sensitive in non-lesional tissue; operator-dependent</u>	<u>Pennisi <i>et al.</i> (2015); Solano-Gallego <i>et al.</i> (2009); Brianti <i>et al.</i> (2020)</u>
<u>Isolation in culture</u> ☒ ☐ ☐ <u>for confirmation of clinical cases</u> <u>Cat</u>	<u>Bone marrow, lymph node, spleen aspirates or skin biopsies; target: viable Leishmania promastigotes cultured in EMTM, NNN, or Schneider's media</u>	<u>Sensitivity: low to moderate (~40–70%) in symptomatic animals with high parasite burden; Specificity: ~100%; no BLCM used in cats</u>	<u>Symptomatic cats in endemic regions; used primarily in research or when parasite typing is needed</u>	<u>Not WOA-validated; described in selected case reports and laboratory studies</u>	<u>Confirms presence of viable parasites; allows further molecular or phenotypic analysis (e.g., drug susceptibility, species typing)</u>	<u>Time-consuming (1–4 weeks), requires invasive sampling and sterile technique; low sensitivity in subclinical or chronic infections</u>	<u>Pennisi <i>et al.</i> (2015); Maia <i>et al.</i> (2015); Brianti <i>et al.</i> (2020); Solano-Gallego <i>et al.</i> (2009)</u>
<u>PCR</u> ☐ ☐ ☐ <u>for contribute to eradication policies,</u> ☒ ☒ ☒ <u>for confirmation of clinical cases,</u> ☒ ☒ ☐ <u>for prevalence of infection – surveillance</u> <u>Cat</u>	<u>Whole blood, lymph node, bone marrow aspirates, skin biopsies; target: Leishmania DNA (kDNA minicircle, SSU rRNA, ITS1 regions)</u>	<u>Sensitivity: high (~85–100%) in symptomatic cats; lower in asymptomatic cats due to intermittent parasitemia; Specificity: >95%; validated against parasitology and serology in some studies</u>	<u>Symptomatic and asymptomatic cats from endemic and non-endemic areas; especially useful in research and targeted screening</u>	<u>Not WOA-validated for Fel.; widely used in clinical and epidemiological studies; standardised in reference labs for canine leishmaniosis</u>	<u>High sensitivity and specificity; detects infection in early or atypical forms; useful in identifying silent carriers; can be used for species identification and genetic typing</u>	<u>Requires specialised lab infrastructure and trained personnel; false negatives possible in chronic or low-parasitaemia cases; cost and sample quality can limit field application</u>	<u>Pennisi <i>et al.</i> (2015); Maia & Campino (2011); Brianti <i>et al.</i> (2020); Solano-Gallego <i>et al.</i> (2009)</u>

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>IFAT</u> ☐ ☐ ☐ <u>for contribute to eradication policies</u> ☒ ☒ ☐ <u>for confirmation of clinical cases, and for prevalence of infection – surveillance.</u> ☒ ☐ ☐ <u>for immune status in individual animals or populations post-vaccination</u> <u>Cat</u>	<u>Serum; target: anti-Leishmania infantum IgG antibodies</u>	<u>Sensitivity: variable (~60–85%) depending on clinical status; Specificity: ~85–98%; no standardised feline cut-off; some ROC analyses in experimental studies</u>	<u>Symptomatic and asymptomatic cats; endemic and non-endemic areas; tested mainly in research and small-scale surveys</u>	<u>Not WOAH-validated in cats; widely used in clinical studies; reference test for serology in canine leishmaniosis</u>	<u>Relatively accessible; semi-quantitative; helpful in detecting exposure; useful in combination with PCR</u>	<u>Lack of standardised cut-off for cats; does not distinguish between vaccination and infection; inter-laboratory variability; possible false negatives in immunosuppressed cats</u>	<u>Pennisi <i>et al.</i> (2015); Solano-Gallego <i>et al.</i> (2009); Brianti <i>et al.</i> (2020); Miró <i>et al.</i> (2021)</u>
<u>ELISA</u> ☐ ☐ ☐ <u>for eradication policies</u> ☒ ☒ ☐ <u>for confirmation of clinical cases, and for prevalence of infection – surveillance.</u> ☒ ☐ ☐ <u>for immune status in individual animals or populations post-vaccination</u> <u>Cat</u>	<u>Serum or plasma; target: anti-Leishmania infantum IgG antibodies (against crude or recombinant antigen)</u>	<u>Sensitivity: ~70–90% depending on disease stage and antigen used; Specificity: ~85–98%; cut-off values in cats are not standardised; some ROC analyses in experimental settings</u>	<u>Symptomatic and asymptomatic cats in endemic areas; used in epidemiological studies and some vaccine response investigations</u>	<u>Not WOAH-validated for cats; evaluated in experimental studies and small-scale field surveys; some commercial kits used off-label</u>	<u>High-throughput, cost-effective for surveillance; adaptable to screening programmes; may detect early or subclinical infections</u>	<u>No standard cut-off for cats; antibodies may not indicate active infection; cross-reactivity possible; limited availability of feline-specific kits</u>	<u>Pennisi <i>et al.</i> (2015); Maia & Campino (2011); Solano-Gallego <i>et al.</i> (2009); Brianti <i>et al.</i> (2020)</u>

MEETING OF THE BIOLOGICAL STANDARDS COMMISSION

Paris, 8–12 September 2025

CHAPTER 3.1.17.

**PARATUBERCULOSIS
(JOHNE'S DISEASE)**

SUMMARY

Description of the disease: *Paratuberculosis (Johne's disease) is a chronic enteritis of ruminants caused by Mycobacterium avium subsp. paratuberculosis (MAP).*

Control of paratuberculosis is difficult because of the prolonged course of infection, the predominantly subclinical nature of the disease and lack of tests for accurate detection of subclinically infected animals.

The diagnosis of paratuberculosis is aimed at two main principal tasks: the diagnosis of clinical disease and the detection of subclinical infection. The latter is essential for control of the disease at the farm, national or international level.

Diagnosis of advanced stage paratuberculosis is made on clinical grounds confirmed by the demonstration-observation of MAP in the faeces by microscopy, or demonstration of MAP by culture, or ~~by the use of DNA probes and the~~ polymerase chain reaction (PCR). Diagnosis is made at necropsy by the finding of the pathognomonic lesions of the disease in the intestines, either grossly with the demonstration of typical acid-fast organisms in impression smears of the lesions or histologically, and by isolation of MAP in culture.

The detection of subclinical infection relies on the detection of specific antibodies by serology, culture of MAP from faeces or tissues collected at necropsy, PCR, or the demonstration of cell-mediated responses. The choice of test depends on the circumstances and the degree of sensitivity and specificity required at individual animal or herd level.

Detection of the agent: ~~*The diagnosis of paratuberculosis is divided into two parts: the diagnosis of clinical disease and the detection of subclinical infection. The latter is essential for control of the disease at the farm, national or international level.*~~

~~*The detection of subclinical infection depends on the detection of specific antibodies by serology, culture of MAP from faeces or tissues collected at necropsy, PCR, or the demonstration of cell-mediated responses. The choice of test depends on the circumstances and the degree of sensitivity and specificity required at individual animal or herd level.*~~

Cultures of MAP may be obtained from faeces or tissues, after treatment to eliminate contaminants, by inoculation into artificial media with and without the specific growth factor – mycobactin – that is essential for the growth of MAP.

End-point or real-time PCR approaches have been developed and validated for the direct detection of MAP DNA in faeces and other matrices, with a trend towards an increased sensitivity compared with cultural assays.

Serological tests: ~~Control of paratuberculosis is difficult because of the prolonged course of infection, the predominantly subclinical nature of the disease and lack of tests for accurate detection of subclinically infected animals.~~

The serological test commonly used for paratuberculosis in cattle is ~~absorbed~~ enzyme-linked immunosorbent assay (ELISA). Agar gel immunodiffusion remains a valuable test for the detection of paratuberculosis in sheep and wild ruminants. Sensitivity and specificity are often determined by reference to results of faecal culture, which itself has unknown sensitivity in subclinically infected cattle. When used to confirm diagnosis of paratuberculosis in cows with typical clinical signs, ELISA performs very well.

Tests for cell-mediated immunity: Interferon-gamma release assay and delayed type hypersensitivity methods (skin test) have been used, but interpretation ~~in both cases~~ is difficult.

Requirements for vaccines and diagnostic biologicals: Vaccines for paratuberculosis may be live attenuated or killed bacteria either incorporated with an adjuvant or lyophilised and adjuvanted on reconstitution. Bacterial counting is difficult and bacterial content of vaccines may be based on weight, while vaccine potency may be judged by batch tests for sensitising ability in guinea pigs.

Vaccine safety or abnormal toxicity may also be tested in guinea pigs.

For diagnostic skin tests, Johnin and avian tuberculin are purified protein derivatives (PPD) of a heat-treated culture of MAP or *M. avium*, respectively. Johnin is standardised for content of PPD by chemical assay and its biological activity is identified in guinea pigs sensitised with MAP. Avian tuberculin activity is determined in guinea pigs sensitised with *M. avium* by comparison with a reference preparation calibrated in international units.

A. INTRODUCTION

1. Nature and classification of the pathogen

Mycobacterium avium subspecies *paratuberculosis* (MAP) is an organism first observed by Johnne & Frothingham in 1895. MAP causes paratuberculosis or Johnne's disease, an intestinal granulomatous infection (Thorel *et al.*, 1990). MAP is an extremely slow 'in-vitro' growing mycobacterium and shares high genome homology with the other subspecies belonging to *M. avium*. Paratuberculosis is was found most often among domestic ruminants (cattle, sheep, goats, camelids and buffaloes) as well as wild ruminants (corvids) and has a global distribution. The disease has also been reported in horses, donkeys, pigs, rabbits, stoats, foxes and weasels (Greig *et al.*, 1999). Under natural conditions, the disease in cattle spreads by ingestion of MAP from the contaminated environment. The disease persists after the introduction of infected animals. Infection can be spread vertically to the fetus (Larson & Kopecky, 1970) and semen can be infected with the organism (Larson & Kopecky, 1970 Sweeney *et al.*, 1995). The primary source of infection in calves is faeces from infected cows or milk that is contaminated with the faeces of diseased cattle. Isolates from sheep have different characteristics from those in cattle, but the laboratory detection and diagnostic methods are the same.

The identification of MAP ~~is was~~ based on its mycobactin requirement and on ~~its association with clinical signs and~~ defined laboratory findings, such as culture and polymerase chain reaction (PCR) results. Mycobactin dependence has long been used as a taxonomic characteristic for MAP because most mycobacteria are able to make mycobactin for themselves. MAP, *M. silvaticum* and some primary isolates of *M. avium* lack this capacity, however, and require mycobactin to grow in the laboratory. Thus, the mycobactin requirement is not confined to MAP; this characteristic exists to various degrees within the *M. avium* ~~group~~ species.

2. Description and impact of the disease

Clinical signs of paratuberculosis are a slowly progressive wasting and diarrhoea, which is intermittent at first, becoming progressively more severe until it is constantly present in bovines. It is also reported as a significant clinical sign in farmed deer. Diarrhoea is less common in small ruminants.

Early lesions occur in the walls of the small intestine and the draining mesenteric lymph nodes, and infection is confined to these sites at this stage. As the disease progresses, gross lesions occur in the ileum, jejunum, terminal small intestine, caecum and colon, and in the mesenteric lymph nodes. MAP is present in the lesions and, terminally, throughout the body. The intestinal lesions are responsible for a protein leak and a protein malabsorption syndrome, which lead to muscle wasting. Clinical signs usually first appear in young adulthood, but the disease can occur in animals at any age over 1–2 years and in dairy cattle is most frequently reported in the 3- to 5-year-old age group.

Within a few weeks of infection, a phase of multiplication of *MAP* begins in the walls of the small intestine. Depending on the natural resistance of the individual, this infection is eliminated, or the animal remains infected as a healthy carrier. The proportion of animals in these categories is unknown. A later phase of multiplication of the organisms in a proportion of carriers leads to the extension of lesions, interference with gut metabolism and clinical signs of disease. Subclinical carriers excrete variable numbers of *MAP* in the faeces. In most cases larger numbers of organisms are excreted as clinical disease develops.

3. Epidemiology and susceptible species

The disease is worldwide diffused and endemic in many countries (Bastida and Juste, 2011; Nielsen and Toft, 2008). Paratuberculosis is found most often among domestic ruminants (cattle, sheep, goats, camelids and buffaloes) as well as wild ruminants (cervids) and has a global distribution. The disease has also been reported in horses, donkeys, pigs, rabbits, stoats, foxes and weasels (Greig *et al.*, 1999). Under natural conditions, the disease in cattle spreads by ingestion of *MAP* from the contaminated environment. The disease persists after the introduction of infected animals. Infection can be spread vertically to the fetus and semen can be infected with the organism (Larson & Kopecky, 1970 Sweeney *et al.*, 1995). The primary source of infection in calves is faeces from infected cows or milk that is contaminated with the faeces of diseased cattle. Isolates from sheep have different characteristics from those in cattle, but the laboratory detection and diagnostic methods are the same.

4. Zoonotic potential and biosafety and biosecurity requirements

MAP organisms and potentially infected specimens should be handled at an appropriate biosafety and containment level determined by biorisk analysis as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities.*

5. Differential diagnosis

Cell-mediated immune responses (CMI) are detectable early in the infection and remain present in a proportion of the subclinically infected carriers, but as the disease progresses, CMI wanes and may be absent in clinical cases. Serum antibodies are detectable later than CMI. They may also be present in animals that have recovered from infection. Serum antibodies are present more constantly and are of higher titre as lesions become more extensive, reflecting the amount of antigen present. In sheep, there may be a serological response that is more likely to be detected in multibacillary than in the paucibacillary form of the disease.

Other mycobacterial diseases and infections, including mammalian and avian tuberculosis, stimulate CMI and the presence of serum antibodies. It follows therefore that these diseases need to be differentiated from paratuberculosis, both clinically and by the use of specific diagnostic tests. Exposure to environmental saprophytic mycobacteria may also sensitise livestock, resulting in nonspecific CMI reactions.

Animals vaccinated against paratuberculosis with whole cell vaccines develop both CMI and serum antibodies. Vaccination is an aid to the prevention of clinical disease, but does not necessarily prevent infection. It also interferes with programmes for the diagnosis and control of bovine tuberculosis. Thus, if it is necessary to attempt a diagnosis of infection in vaccinates, it is advisable to use tests detecting the antigen *MAP* in samples of faeces or tissues.

In individual animals, especially from a farm in which the disease has not previously been diagnosed, a tentative clinical diagnosis must be confirmed by laboratory tests. However, a ~~definitive-presumptive~~ diagnosis may be warranted on clinical grounds alone if the clinical signs are typical and the disease is known to be present in the herd. Confirmation of paratuberculosis depends on the finding of either gross lesions with the demonstration of typical acid-fast organisms in impression smears or microscopic pathognomonic lesions and the isolation in culture of *MAP*.

~~*MAP* organisms and potentially infected specimens should be handled at an appropriate biosafety and containment level determined by biorisk analysis as described in 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 diagnosis of paratuberculosis 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)						
Histopathology*	☑☑☑ ☐☐☐	☐☐☐	☑☐☐	☑☑☑	☐☐☐☑☐☐	☐☐☐
Faecal ZN staining	☐☐☐	☐☐☐	☐☐☐	☑☐☐	☐☐☐☑☐☐	☐☐☐
Culture	☐☐☐-☑☐☐	☑☐☐	☑☐☐	☑☑☑	☑☐☐ ☑☑☐	☐☐☐
PCR	☑☑☑-☑☑☐	☑☑☑-☑☑☐	☑☐☐-☑☑☑	☑☑☐-☑☑☑	☑☐☐-☑☑☑	☐☐☐
Detection of immune response^(h)						
AGID**	☑☑☐	☑☐☐	☑☐☐	☑☑☐	☑☑☑-☑☑☐	☑☑☑-☑☑☐
<u>Serum ELISA</u>	☑☑☑-☑☑☐	☑☑☑-☑☑☐	☑☑☑	☑☐☐-☑☑☑	☑☑☑	☑☑☑-☑☑☐
<u>Milk tank ELISA</u>	☑☑☐	☐☐☐	☑☑☑	☐☐☐	☑☑☑	☐☐☐
IFN-γ release assay	☐☐☐-☑☐☐	☐☐☐-☑☐☐	☑☐☐	☐☐☐	☐☐☐	☑☑☑-☑☑☐
DTH	☐☐☐	☐☐☐-☑☐☐	☑☐☐	☐☐☐	☐☐☐	☑☑☑-☑☑☐

Key: ☑☑☑ = recommended for this purpose; ☑☑☐ recommended but has limitations;

☑☐☐ = suitable in very limited circumstances; ☐☐☐ = not appropriate for this purpose.

* = post-mortem use only; ** = appropriate for the use in sheep, goats and wild animals.

ZN = Ziehl–Neelsen; PCR = polymerase chain reaction; AGID = agar gel immunodiffusion; ELISA = enzyme-linked immunosorbent assay; IFN-γ = gamma interferon; DTH = delayed-type hypersensitivity.

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

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

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

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

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

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

As it is expected that vaccinated animals will show a positive, albeit variable, response to serological techniques, individual serological results cannot reliably determine whether a positive result in an animal is due to infection or prior vaccination. Consequently, test results at the individual level are of limited value in distinguishing between infection and vaccination. At the herd level, however, aggregating results increases reliability and, under specific circumstances, allows more confident inferences to be made about whether a herd has been vaccinated

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

^(h)A combination of the listed serological tests is recommended.

To diagnose the presence of paratuberculosis in an individual clinically suspect animal, a number of laboratory tests can be used including: faecal smears, faecal and tissue culture, DNA ~~probe~~ probe amplification approaches (PCR) using faeces or tissues, serology, necropsy and histology (Table 1).

Herd tests to detect subclinical infection are carried out to determine the prevalence of the infection, usually so that control measures can be instituted. In general, paratuberculosis assays used to detect infected animals showed poor sensitivity and high specificity, with sensitivity varying depending on the stage of the disease. As no test is 100% sensitive or specific, control of the disease by the disposal of positive reactors depends on repeated tests at 6-month or yearly intervals over a number of years and the elimination of reactors to serological tests or faecal shedders; the removal of offspring from female reactors is also considered to be prudent. Even these procedures are not always successful without changes in hygiene and livestock management to reduce the transmission of infection within a herd. Similar test strategies with repeated tests at the herd level can also be applied within control

programmes to estimate herd-level probabilities of freedom from infection, and thus to identify low risk herds for safer trade.

1. Detection of the agent

1.1. Necropsy

Paratuberculosis cannot be diagnosed on superficial examination of the intestines for signs of thickening. The intestines should be opened from the duodenum to the rectum to expose the mucosa. There is not always a close correlation between the severity of clinical signs and the extent of intestinal lesions. The mucosa, especially of the terminal ileum, is inspected for pathognomonic thickening and corrugation. Early lesions are seen by holding the intestine up to the light, when discrete plaques can be visualised. Mucosal hyperaemia, erosions and petechiation have been observed in deer with paratuberculosis. The earliest lesions are thickening and cording of lymphatics. The mesenteric lymph nodes are usually enlarged and oedematous. Caseous and/or calcified lesions in mesenteric lymph nodes are often seen in goats and to a lesser extent in sheep (~~Fodstad & Gunnarsson, 1979~~). Smears from the affected mucosa and cut surfaces of lymph nodes can be stained by Ziehl–Neelsen’s method and examined microscopically for acid-fast organisms that have the morphological characteristics of *MAP*. However, acid-fast organisms are not present in all cases. Diagnosis is therefore best confirmed by the collection of multiple intestinal wall and mesenteric lymph node samples into fixative (10% formol saline) for subsequent histology. Both haematoxylin-and-eosin-stained sections and Ziehl–Neelsen-stained sections should be examined. The typical lesions of paratuberculosis consist of infiltration of the intestinal mucosa, submucosa, Peyer’s patches and the cortex of the mesenteric lymph nodes with large macrophages, also known as epithelioid cells, and multinucleate giant cells, in both of which clumps or singly disposed acid-fast bacilli are usually, but not invariably, found.

1.2. Bacteriology (microscopy)

Ziehl–Neelsen-stained smears of faeces or intestinal mucosa are examined microscopically. A presumptive diagnosis of paratuberculosis can be made if clumps (three or more organisms) of small (0.5–1.5 µm), strongly acid-fast bacilli are found. The presence of single acid-fast bacilli in the absence of clumps indicates an inconclusive result. The disadvantages of this test are that it does not differentiate among other mycobacterial species and only a small proportion of cases can be confirmed on microscopic examination of a single faecal sample.

1.3. Bacteriology (culture)

The isolation of *MAP* from an animal provides the definitive diagnosis of infection with the organism. Although culture is technically difficult and time-consuming to carry out, it is the only test that does not produce false-positive results, if coupled with other method to confirm the identification of *MAP* (100% specificity). However, due to the potential pass-through phenomenon, it is theoretically possible that faecal culture testing of non-infected animals on contaminated premises can lead to false-positive reactions (Nielsen & Toft, 2008).

~~The~~ Faecal culture ~~is~~ has been widely considered to be the gold standard for the diagnosis of paratuberculosis in live animals (affected animals) for decades, but currently this assumption has been called into question based on the results obtained by PCR approaches worldwide. Actually, the Faecal culture can detect most animals in the advanced stages of the disease but identifies only a few animals in the early stages of infection (Nielsen & Toft, 2008). According to conditions, the sensitivity of faecal culture is 70% for affected cattle, 74% for infectious cattle and 23–29% for infected cattle.

There are several culture methods, which vary with respect to media and sample processing protocols. The cultivation of *MAP* is always performed using special media supplemented with mycobactin J, which is available commercially.

MAP organisms are vastly outnumbered by other bacteria or fungi in faecal and intestinal tissue specimens. The successful isolation of *MAP* from such samples depends on efficient inactivation of these undesirable organisms. The optimal method of decontamination must have the least inhibitory effect on growth of *MAP*. Routine decontamination protocols were shown to decrease the number of *MAP* organisms isolated per sample by about 2.7 log₁₀ and 3.1 log₁₀ for faeces and tissues, respectively (Reddacliff *et al.*, 2003).

There are two basic methods of sample decontamination: the method using oxalic acid and NaOH for decontamination and Löwenstein–Jensen (LJ) medium for growth, and the method using hexadecylpyridinium chloride (HPC) for decontamination in combination with solid media such as Herrolds’s egg yolk medium (HEYM) or Middlebrook 7H10 and liquid media such as Middlebrook 7H9 or

commercial equivalents for growth. Although it has been published that HEYM supports growth of bovine isolates of *MAP* significantly better than LJ, recent studies have shown that certain strains grow better on LJ or Middlebrook media. The advantage of 7H10 medium is that it better supports the growth of ovine strains compared with HEYM. Liquid media have been reported to be more sensitive than solid media for both ovine and bovine strains.

Automated monitoring systems have been developed for the cultivation of *MAP* in liquid culture, offering the advantage of avoiding the lecture phase, which is continually monitored in order to evaluate O₂ consumption or gas pressure. Drawbacks of these systems are the high cost of reagents needed to perform the test as well as the need for standardisation of the procedures. Notably, the output of these systems requires confirmatory testing to avoid false positive results.

Primary colonies of *MAP* on solid media may be expected to appear any time from 5 weeks to 6 months after inoculation. Sheep strains, including the uncommon, bright yellow pigmented types, grow less well than cattle strains on commonly used media such as HEYM or LJ, and primary cultures should not be discarded as negative without prolonged incubation. ~~The solid medium Middlebrook 7H10 supplemented with egg yolk and mycobactin is excellent for the cultivation of ovine strains of *MAP* (Whittington *et al.*, 1999).~~

Primary colonies of the cattle strain of *MAP* on HEYM are very small, convex (hemispherical), soft, non-mucoid and initially colourless and translucent. Colony size is initially pinpoint. It may remain at 0.25–1 mm and tend to remain small when colonies are numerous on a slope. Colony margins are round and even, and their surfaces are smooth and glistening. The colonies become bigger more raised, opaque, off-white cream to buff or beige coloured as incubation continues. Older isolated colonies may reach 2 mm. The colonial morphology changes with age from smooth to rough, and from hemispherical to mamillate.

On modified 7H10 medium, colonies of the cattle strain are less convex than those on HEYM, especially in aged cultures. They are pinpoint to approximately 1 mm in diameter and, being buff coloured, are only slightly lighter than the media. Compared with colonies of cattle strains on HEYM, those on 7H10 are more difficult to detect. Colonies of the sheep strain of *MAP* on modified 7H10 are convex, soft, moist, glistening, off-white to buff, and very similar to the colour of the media. Colonies are typically between pinpoint and 0.5 mm, but can reach 1 mm, and rarely 1.5 mm if few colonies occur on a slope.

Saprophytic mycobacteria may have a similar appearance on either medium but are often evident after 5–7 days.

For identification of *MAP*, small inoculum of suspect colonies should be subcultured on the same medium with and without mycobactin, to demonstrate mycobactin dependency. Mycobactin is present in the cell wall of the organism, and heavy inoculum may contain enough mycobactin to support the growth of *MAP* on medium that contains no mycobactin. In addition, to finalise the correct diagnosis, PCR confirmation (targeting IS900 or F57) should be used to confirm the identification of *MAP* isolates.

1.3.1. Media

Examples of suitable media are:

i) Herrold's egg yolk medium with mycobactin and pyruvate

For 1 litre of medium: 9 g peptone; 4.5 g sodium chloride; 2.7 g beef extract; 27 ml glycerol; 4.1 g sodium pyruvate; 15.3 g agar; 2 mg mycobactin; 870 ml distilled water; six egg yolks (120 ml); and 5.1 ml of a 2% aqueous solution of malachite green. Measure the first six ingredients and dissolve by heating in distilled water. Adjust the pH of the liquid medium to 6.9–7.0 using 4% NaOH, and test to ensure the pH of the solid phase is 7.2–7.3. ~~Add the mycobactin dissolved in 4 ml ethyl alcohol.~~ Autoclave at 121°C for 25 minutes. Cool to 56°C and aseptically add six sterile egg yolks¹ ~~and~~ sterile malachite green solution and mycobactin dissolved in 4 ml ethyl alcohol. Blend gently and dispense into sterile tubes. Note that the batch coming out of the autoclave is divided to add mycobactin to one part and not to the other. In this way, two identical batches of medium remain, with and without mycobactin. This helps to compare the growths.

¹ Use fresh eggs not more than 2 days old from a flock that is not receiving antibiotics. With a brush, scrub the eggs with water containing a detergent. Rinse with water and place the eggs in 70° alcohol for 30 minutes. Dry by inserting between two sterile towels. With sterile rat-tooth forceps, crack one end of the eggshell, making a hole of approximately 10 mm, and remove the egg white with the forceps and gravity. Make the hole larger and break the yolk. Mix the egg yolk by twirling the forceps, and remove the yolk sac. Pour the mixed egg yolk into media.

It is permissible to add 50 mg chloramphenicol, 100,000 U penicillin and 50 mg amphotericin B.

ii) Modified Dubos's medium

For 1 litre of medium: 2.5 g casamino acids; 0.3 g asparagine; 2.5 g anhydrous disodium hydrogen phosphate; 1 g potassium dihydrogen phosphate; 1.5 g sodium citrate; 0.6 g crystalline magnesium sulphate; 25 ml glycerol; 50 ml of a 1% solution of Tween 80; and 15 g agar. Dissolve each salt in distilled water with minimum heat and make up to 800 ml. Add mycobactin in alcoholic solution at 0.05% (2 mg dissolved in 4 ml ethyl alcohol), heat the medium to 100°C by free-steaming, and then sterilise by autoclaving at 115°C for 15 minutes. Cool to 56°C in a water bath, add antibiotics (100,000 U penicillin; 50 mg chloramphenicol; and 50 mg amphotericin B) and serum (200 ml of bovine serum sterilised by filtering through a Seitz 'EX' pad and inactivated by heat at 56°C for 1 hour). The medium is kept thoroughly mixed and then dispensed into sterile tubes. An advantage of this medium is that it is transparent, which facilitates the early detection of colonies.

iii) Modified Middlebrook 7H10 and 7H9

Middlebrook media with added Mycobactin and various commercially available supplements can be used. Further advice on formulation can be obtained from the WOA Reference Laboratories.

iv) Löwenstein–Jensen medium with or without mycobactin

1.3.2. Sample preparation

i) Processing tissue specimens

Chemical preservatives should not be used. The tissues can be frozen at –70°C.

To avoid contamination, the faeces should be rinsed from portions of intestinal tract before shipment to the laboratory.

a) Digestion/sedimentation method for decontamination of tissues

Approximately 4 g of mucosa from the ileocaecal valve or 4 g of mesenteric node are placed in a sterile blender jar containing 50 ml of trypsin (2.5%). The mixture is adjusted to neutrality using 4% NaOH and pH paper and stirred for 30 minutes at room temperature on a magnetic mixer. The digested mixture is filtered through gauze. The filtrate is centrifuged at approximately 2000–3000 *g* for 30 minutes. The supernatant fluid is poured off and discarded. The sediment is resuspended in 20 ml of 0.75% HPC and allowed to stand undisturbed for 18 hours at room temperature. The particles that settle to the bottom of the tube are to be used as the inoculum and are removed by pipette without disturbing the supernatant fluid. Alternatively, other methods of decontamination can be used, such as treatment with 5% oxalic acid.

b) Double incubation method for decontamination of tissues

About 2 g of tissue sample (trimmed of fat) is finely chopped using a sterile scalpel blade or scissors and homogenised in a stomacher for 1 minute in 25 ml 0.75% HPC. Allow the sample to stand so that foam dissipates and larger pieces of tissue settle. Pour tissue homogenate into a centrifuge tube taking care to avoid carry over of fat or large tissue pieces. Allow to settle for 30 minutes then take 10 ml of the suspension from just above the sediment to a 30 ml tube and incubate for 3 hours at 37°C. Centrifuge for 30 minutes at 900 *g*, discard supernatant fluid and resuspend pellet in 1 ml antibiotic cocktail containing 100 µg of each of vancomycin, amphotericin and nalidixic acid (VAN). Incubate overnight at 37°C. Use the suspension to inoculate media as described below.

c) Inoculation of culture media and incubation

Approximately 0.1 ml of inoculum is transferred to each of three slants of Herrold's medium containing mycobactin and to one slant of Herrold's medium without mycobactin. The inoculum is distributed evenly over the surface of the slants. The tubes are allowed to remain in a slanted position at 37°C for approximately 1 week with screw caps loose. The tubes are returned to a vertical position when the free moisture has evaporated from the slants. The lids are tightened, and the tubes are placed in baskets in an incubator at 37°C.

The egg in Herrold's medium contributes sufficient phospholipids to neutralise the bactericidal activity of residual HPC in the inoculum. The other media (Modified Dubos and Middlebrook) do not have this property. Other treatments can be used for sample decontamination, for example oxalic acid at 5%.

HPC is relatively ineffective in controlling the growth of contaminating fungi. Amphotericin B (fungizone) was found to control effectively fungal overgrowth of inoculated media. Fungizone may be incorporated in the Herrold's medium at a final concentration of 50 µg per ml of medium. Due to loss of antifungal activity, storage of Herrold's medium containing fungizone should be limited to 1 month at 4°C.

The slants are incubated for at least 4 months and observed weekly from the sixth week onwards.

ii) Processing faecal specimens

No chemical preservative is used. The faecal specimens can be frozen at -70°C.

a) Suspension and decontamination of faeces

4–5–10 g of faeces is transferred to a 50 ml tube containing 20 ml of sterile distilled water. The mixture is shaken for 30 minutes at room temperature. The larger particles are allowed to settle for 30 minutes. The uppermost 5 ml of faeces suspension is transferred to a 50 ml tube containing 20 ml of 0.95% HPC. The tube is inverted several times to assure uniform distribution and allowed to stand undisturbed for 18 hours at room temperature.

b) Inoculation of culture media

0.1 ml of the undisturbed sediment is transferred to each of four slants of Herrold's medium, three with mycobactin and one without mycobactin. A smear may be made from the sediment and stained by the Ziehl–Neelsen method.

c) Incubation and observation of slants

The same as for tissue specimens.

Variations in the above methods have been described (Collins *et al.*, 1990; Morkal *et al.*, 1968 reviewed in Dane *et al.*, 2023). The sensitivity of culture may be enhanced using liquid media and with centrifugation rather than sedimentation techniques. The double incubation method described by Whitlock *et al.* (1991) assists with decontamination of the inoculum and offers higher sensitivity than the sedimentation or filtration protocols (Eamens *et al.*, 2000). The double incubation method involves mixing 2 g faeces with 45–35 ml saline or water followed by sedimentation for 30 minutes and transferring (avoiding fibrous matter) the top 5 ml of the suspension to 25 ml of 0.9% HPC in half-strength brain–heart infusion. After incubating at 37°C for 16–24 hours, the mixture is centrifuged at 900 g for 30 minutes (room temperature), the supernatant is discarded, and the pellet is resuspended in 1 ml VAN. The mixture is incubated for 24–72 hours at 37°C and used to inoculate media as described above.

1.4. Molecular methods – detection of nucleic acids ~~DNA probes and polymerase chain reaction~~

PCR assays for the detection of MAP DNA have two stages: DNA extraction from different matrices (faeces, milk, tissue or others) followed by DNA amplification. DNA extraction methods can be broadly divided into manual methods, using spin columns or other capture DNA reagents, and semi-automated methods, most of which use magnetic devices capable of capturing magnetic beads with high affinity for DNA. Of key importance is that the DNA extraction method to be employed includes a mechanical lysis step as well as the chemical lysis. If possible, introducing an internal amplification control for each sample can be pivotal to differentiate between inhibited and true negative samples.

Extraction of nucleic acids

Different kits and homemade procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid is important and can be checked using a suitable method as appropriate to the circumstances.

Real-time PCR

~~DNA probes are being developed that offer a means of detecting MAP in diagnostic samples and of rapidly identifying bacterial isolates (Ellingson *et al.*, 1998). They have been used to distinguish between MAP and other mycobacteria.~~

McFadden *et al.* have identified a sequence (McFadden *et al.*, 1987), termed IS900, which is An insertion sequence specific for MAP, termed IS900, has been described (Vary *et al.*, 1990), which is the DNA target currently used in most of the PCR assays aimed at detecting MAP DNA. The sequence is present in a variable number of copies in the MAP genome. However, it has been reported that a small number of isolates other than MAP have produced amplified products the same size as expected from MAP. A restriction enzyme digest or sequencing may be applied to positive IS900 products to confirm that their sequence is consistent with MAP.

The identifications of new DNA sequences considered to be unique to MAP (ISMap2, f57, and ISMap02 sequences), offer additional tools for rapid identification of this organism using the PCR technology (Stabel & Bannantine, 2005; Strommenger *et al.*, 2001; Vansnick *et al.*, 2004). The restriction enzyme analysis of IS1311, an insertion sequence common to *M. avium* subsp. *avium* and MAP can be used to distinguish between these species and for typing of ovine, bovine and bison strains of MAP (Sevilla *et al.*, 2005; ~~Whittington *et al.*, 1998~~).

In recent years, real-time PCR methods have been extensively developed to detect MAP from different specimens (blood, milk, faeces, tissues and environmental samples). The technique is rapid and offers hope for detection of fastidious and slow growing microorganisms, such as MAP. However, this molecular tool is greatly influenced by the quality of nucleic acid samples. Therefore, a DNA extraction method that provides a DNA sample of sufficient quality and maximum bacterial DNA recovery, coupled with internal amplification controls during the amplification reaction, is a critical step in the use of real-time PCR (~~Parka *et al.*, 2014~~).

PCR assays have improved to the point where they play an important role in the diagnosis of disease (~~Leite *et al.*, 2013~~). A recent systematic review and meta-analysis (Liu *et al.*, 2025) estimated the accuracy of real-time PCR assays for detecting paratuberculosis relative to culture methods. The pooled sensitivity was 0.92 (95% CI 0.85–0.96) and the specificity 0.85 (95% CI 0.77–0.91). One assay (Russo *et al.*, 2022, see table below) targeting IS900 has also been validated according to the Chapter 1.1.6. Principles and methods of validation of diagnostic assays for infectious diseases of terrestrial animals. The estimated diagnostic sensitivity and specificity, evaluated by the latent class approach varied according to the real-time PCR cut-off values chosen as well as the prevalence scenarios considered. Commercial diagnostic PCR tests for the detection of MAP in milk and faecal samples are available, but users should consider the interpretation of acquired data and fitness for purpose before adopting such methods.

Primers and probes (sequences) of a validated real-time PCR method

<u>Pathogen/ target gene</u>	<u>Primer/probe (5'–3')</u>	<u>Concentration</u>	<u>Cycling parameters^(a)</u>
<u>Method 1: Russo <i>et al.</i> (2022)</u>			
<u>MAP/IS900</u>	IS900 Fwd real-time PCR: CCG-GTA-AGG-CCG-ACC-ATT-A IS900 Rev real-time PCR: ACC-CGC-TGC-GAG-AGC-A IS900 Probe real-time PCR: CAT-GGT-TAT-TAA-CGA-CGA-CGC-GCA-GC	300 nM 300 nM 150 nM	40 cycles of: 95°C/15 sec and 60°C/60 sec

^(a)A denaturation step prior to cycling has not been included. The denaturation step occurs usually at 95°C for 1–5 minutes or even more if using a hot start DNA polymerase

2. Serological tests

The serological test commonly used for paratuberculosis in cattle is enzyme-linked immunosorbent assay (ELISA). The ~~complement fixation test (CFT) and~~ agar gel immunodiffusion (AGID) shows poorer sensitivity and specificity and are no longer recommended in cattle. However, AGID remains a valuable test for the detection of paratuberculosis in ~~sheep~~ animals in which no specific ELISAs have been developed. There is no international reference serum.

2.1. Enzyme-linked immunosorbent assay

The ELISA is, at present, the most sensitive and specific test for serum antibodies to MAP in cattle. ~~Its sensitivity is comparable with that of the CFT in clinical cases, but is greater than that of the CFT in subclinically infected carriers.~~ The specificity of the ELISA is increased by *M. phlei* absorption of sera. The absorbed ELISA, designed by Yokomizo *et al.* (1983; 1985) and modified by Milner *et al.* (1988), was developed into a commercial kit by Cox *et al.* (1991).

The ELISA detects about 30–40% cattle identified as infected by culture of faeces on solid media (Whitlock *et al.*, 2000). Similarly to the culture methods, the sensitivity of the ELISA depends on the level of *MAP* shedding in faeces and the age of animals. A large study performed in Australia showed that the actual sensitivity of the ELISA in 2-, 3- and 4-year-old cows was 1.2%, 8.9% and 11.6%, respectively, but remained between 20 and 30% in older age-groups (Jubb *et al.*, 2004). The overall actual sensitivity for all age-groups was calculated to be about 15% (Jubb *et al.*, 2004; Whitlock *et al.*, 2000). In cattle, the sensitivities of ELISA are in the range 7–94%, and the specificities of ELISA are in the range 40–100% (Nielsen & Toft, 2008).

In small ruminants the commercially available ELISA had a specificity of 98.2–99.5% (95% confidence intervals [CI]) and detected 35–54% (95% CI) of animals with histological evidence of infection (Hope *et al.*, 2000). In another study, the estimated specificity of an in-house ELISA was 99% and its sensitivity measured against histological results was 21.9% (Sergeant *et al.*, 2003). In small ruminants, the sensitivities of ELISA are in the range 16–100%, and the specificities of ELISA are in the range 79–100% (Nielsen & Toft, 2008).

The absorbed ELISA combines the sensitivity of ELISA with the added specificity of an absorption step. Sera to be tested are diluted with buffer containing soluble *M. phlei* antigen prior to testing in an indirect ELISA. This procedure eliminates nonspecific cross-reacting antibodies. In early versions, sera were absorbed with whole *M. phlei*, which were removed by centrifugation prior to testing.

A microtitre plate format has been developed in which *MAP* antigen is coated on to 96-well plates. Samples are diluted in sample diluent containing *M. phlei* to remove cross-reacting antibodies. On incubation of the diluted sample in the coated well, antibody specific to *MAP* forms a complex with the coated antigens. After washing away unbound materials from the wells, horseradish peroxidase (HRPO)-labelled anti-bovine immunoglobulin is added. This reacts with immunoglobulins bound to the solid-phase antigen. The rate of conversion of substrate is proportional to the amount of bound immunoglobulin. Subsequent colour, measured spectrophotometrically (at the wavelength appropriate to the chromogen used), is proportional to the amount of antibody present in the test sample. Several absorbed ELISA kits are commercially available. The method and test materials needed, the interpretation of the results and calculations are fully described in the instructions accompanying the commercial kit. It has been reported that several commercially available ELISAs have similar sensitivities and specificities. Some commercial kits offer an option of testing milk samples. The ELISA on bovine and caprine milk has been found to have specificity similar to that of the serum ELISA, but less sensitive than the blood test (Hendrick *et al.*, 2005; Salgado *et al.*, 2005). In cattle, the sensitivities of milk ELISA are in the range 21–61%, and the specificities of milk ELISA are in the range 83–100% (Nielsen & Toft, 2008).

The ELISA detects about 30–40% of cattle identified as infected by culture of faeces on solid media (Whitlock *et al.*, 2000). Similarly to the culture methods, the sensitivity of the ELISA depends on the level of *MAP* shedding in faeces and the age of the animals. A large study performed in Australia showed that the actual sensitivity of the ELISA in 2-, 3- and 4-year-old cows was 1.2%, 8.9% and 11.6%, respectively, but remained between 20 and 30% in older age-groups (Jubb *et al.*, 2004). The overall actual sensitivity for all age-groups was calculated to be about 15% (Jubb *et al.*, 2004; Whitlock *et al.*, 2000). In cattle, the sensitivities of ELISA varies in the range 7–94%, depending on the stage of infection and the specificities of ELISA are in the range 40–100% (Nielsen & Toft, 2008). At the herd level, milk and serum ELISA characteristics (sensitivity and specificity) varied according to the test protocol used (Field *et al.*, 2022). Cross reactions after the intradermal comparative cervical tuberculin test has been reported (Kennedy *et al.*, 2014). The impact of such cross reactions should be evaluated for each programme, for example, in the UK, the impact on paratuberculosis control programmes at the national level in the cattle population seems to be negligible (Caldow *et al.*, 2023).

In small ruminants, the sensitivities of ELISA are in the range 16–100%, and the specificities of ELISA are in the range 79–100% (Nielsen & Toft, 2008).

2.2. Complement fixation test

The CFT has been the standard test used for cattle for many years. The CFT works well on clinically suspect animals, but does not have sufficient specificity to enable its use in the general population for control purposes. Thus, the CFT is not recommended for control purposes nor for individual animal testing prior to international movement. A variety of CFT procedures are used internationally. There are no international pattern sera with standardised complement fixation units for use as a reference. An example of a microtitre method for performing the CFT is given.

2.1.1. Test procedure

- i) ~~The antigen is an aqueous extract of bacteria from which lipid has been removed (strain *M. paratuberculosis* 316F). *Mycobacterium avium* D9 may also be used.~~
- ii) ~~All sera are inactivated in the water bath at 60°C for 30 minutes and diluted at 1/4, 1/8 and 1/16. A positive control serum and a negative control serum should be included on each plate. The following controls are also prepared: antigen control, complement control and haemolytic system control.~~
- iii) ~~Reconstituted, freeze-dried complement is diluted to contain six times H_{50} (50% haemolysing dose) as calculated by titration against the antigen.~~
- iv) ~~Sheep erythrocytes, 2.5%, are sensitised with 2 units of H_{100} haemolysin.~~
- v) ~~All dilutions and reagents are prepared in calcium/magnesium veronal buffer; 25 µl volumes of each reagent are used in 96-well round-bottom microtitration plates.~~
- vi) ~~Primary incubation is at 4°C overnight and secondary incubation is at 37°C for 30 minutes.~~
- vii) ~~**Reading and interpreting the results:** Plates may be left to settle or centrifuged and read as follows: 4+ = 100% fixation, 3+ = 75% fixation, 2+ = 50% fixation, 1+ = 25% fixation and 0 = complete haemolysis. The titre of test sera is given as the reciprocal of the highest dilution of serum giving 50% fixation. A reaction of 2+ at 1/8 is regarded as positive. Results should be interpreted in relation to clinical signs and other laboratory findings.~~

2.23. Agar gel immunodiffusion test

The AGID test is useful for the confirmation of the disease in clinically suspect cattle, sheep, and goats and wild animals. ~~It has been reported that in small ruminants in New Zealand and Australia the AGID offers slightly higher sensitivity and specificity than that obtained by the ELISAs (Gwozdz *et al.*, 2000; Hope *et al.*, 2000; Sergeant *et al.*, 2003). The reported specificity and sensitivity of the AGID measured against histological histopathology or culture results from the terminal ileum and associated lymph nodes was results were 99–100% (95%CI) for both goats and sheep and the sensitivity was 38–56–14–40% (95% CI), respectively (Hope *et al.*, 2000 Gumber *et al.*, 2006).~~

The antigen employed is a crude protoplasmic extract of laboratory strain *M. avium* 18 (formally *M. paratuberculosis* 18) prepared by disruption of cells in a hydraulic press cell fractionator. Disrupted cells are centrifuged at 40,000 g for 2 hours to remove cell wall debris, and the supernatant fraction is retained and lyophilised. This antigen is resuspended in water at a concentration of 10 mg/ml.

Agarose is dissolved in barbital buffer, pH 8.6, containing sodium azide, to give a final agarose concentration of 0.75%. Agarose may be poured into Petri dishes or on to glass slides. Wells are cut in a hexagonal pattern. Wells are 4 mm in diameter, 4 mm apart, and the agar should be 3–4 mm deep. Antigen is added to centre wells. Test, positive and negative control sera are added to alternate peripheral wells.

Plates are incubated in a humid chamber at room temperature. Gels are examined for precipitation lines after 24 and 48 hours' incubation. The appearance of one or more clearly definable precipitation line(s), showing identity with that of a control positive serum, before or at 48 hours, constitutes a positive test result. Absence of any precipitation lines is recorded as a negative test result. Nonspecific lines may occur.

Several variations of the method are in use.

3. Tests for cell-mediated immunity

The detection of a systemic cell-mediated response precedes detectable antibody production. Animals that are minimally infected frequently fail to react on serological testing but may react positively to tests that measure CMI. In infected populations, a much higher number of animals is expected to react in tests for CMI compared with antibody tests, as CMI is indicative of exposure while antibodies indicate progress of infection.

3.1. Interferon-gamma release assay

The assay is based on the release of gamma interferon (IFN- γ) from sensitised lymphocytes during an 18–36-hour incubation period with specific antigen (avian purified protein derivative [PPD] tuberculin, bovine PPD tuberculin or Johnin²). The quantitative detection of bovine IFN- γ is carried out with a

² Johnin can be obtained from ID-Lelystad, The Netherlands

sandwich ELISA that uses two monoclonal antibodies to bovine IFN- γ . A commercial diagnostic test based on the detection of IFN- γ has been developed for the diagnosis of bovine tuberculosis. The method and test materials needed are fully described in the instructions accompanying the commercial kit. This test has not been validated by the manufacturer for the diagnosis of paratuberculosis. As such, results derived from this assay are frequently difficult to interpret because there is no agreement with respect to the interpretation criteria and types and amounts of antigens used to stimulate blood lymphocytes. In cattle the reported specificity of the test varied from 94% to 67% and the sensitivity varied from 13% to 85%, depending on the interpretation criteria (Kalis *et al.*, 2003; Nielsen & Toft, 2008). A longitudinal study evaluated the accuracy of IFN- γ in bovines (Corneli *et al.*, 2021). The sensitivity and specificity proved to vary according to the cut-off values selected. This publication offers some suggestions on the appropriateness of this method for the diagnosis of paratuberculosis.

Several ELISA kits are commercially available for quantitative detection of IFN- γ on bovine, ovine and caprine plasmas.

3.2. Delayed-type hypersensitivity

The skin test for delayed-type hypersensitivity (DTH) is a measure of cell-mediated immunity, but has limited value. The test is carried out by the intradermal inoculation of 0.1 ml of antigen into a clipped or shaven site, usually on the side of the middle third of the neck. In the past, avian PPD tuberculin or johnin was used for this purpose as it was believed that avian tuberculin and johnin are of comparable sensitivity and specificity. The skin thickness is measured with calipers before and 72 hours after inoculation. Increases in skin thickness of over 2 mm should be regarded as indicating the presence of DTH. It should be noted that positive reactions in deer may take the form of diffuse plaques rather than discrete circumscribed swellings, thus making reading of the test more difficult. The presence of any swelling should be regarded as positive in this species. However, sensitisation to the *M. avium* complex is widespread in animals, and neither avian tuberculin nor johnin are highly specific. Furthermore, the interpretation of the skin test results is complicated by the lack of agreement with respect to interpretation criteria. In a study in which johnin was used to test cattle, the skin test specificity was 88.8% at the cut-off value of ≥ 2 mm, 91.3% at the cut-off value of ≥ 3 mm and 93.5% at the cut-off value of ≥ 4 mm (Kalis *et al.*, 2003). The effect of these cut-off values on the sensitivity has not been determined. The performance of this test may also be significantly affected by minor antigenic differences that occur in different batches of antigen (Kalis *et al.*, 2003). Further research is required to increase the value of the skin test.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

C1. Vaccines

1. Background

Vaccination may interfere with eradication programmes based on immunological testing and elimination of animals identified as infected and can interfere with the interpretation of DTH skin tests for bovine tuberculosis. Despite many efforts to develop new vaccines based on multi- or single epitopes, as well as those derived from attenuated mutants, all the few commercial vaccines now available are exclusively inactivated (killed) whole-cell-based vaccines. Moreover, to date, effective DIVA (differentiating infected from vaccinated animals) strategies are not available. Vaccination can curtail the clinical incidence of paratuberculosis, defer the onset of the disease, and diminish faecal shedding of MAP, thereby mitigating economic losses and transmission of the disease (Bastida & Juste, 2011). Nevertheless, the practice of vaccinating cattle or small ruminants is not allowed in many some countries mainly due to the fear of giving rise or permitted solely under specified circumstances. This is primarily attributable to false positive results in interference with the skin test for bovine tuberculosis.

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

2.1.1. Quality criteria

Purity tests should be carried out on seed cultures and final harvest by stained smears.

2.1.2. Validation as a vaccine strain

Seed strains should be of a prevalent type, which may be checked by biotyping or genetic analysis. They should have been demonstrated to be innocuous when administered by the recommended route of vaccination to intended target species.

2.2. Method of manufacture

2.2.1. Procedure

For vaccine batches, the organisms may be grown on a liquid synthetic medium, such as Reid's synthetic medium. The organisms grow as a pellicle on the liquid surface. To ensure a good surface area, it is convenient to use vessels such as conical flasks containing one-third of their nominal volume of liquid medium. These flasks may be seeded directly from potato slant cultures, but with some strains, one or more passages on liquid medium may be necessary to ensure adequate pellicle growth for the final, vaccine batch passage. Such passaging should usually take place at 2-week intervals as longer periods may result in over-maturation and sinking of the pellicle. Incubation is at 37°C.

To prepare the vaccine, the pellicle growth from 2-week-old cultures of each strain to be included may be separated from the liquid medium by decantation, filtration and pressing between filter paper pads. The moist *MAP* culture is blended with an adjuvant, such as liquid paraffin, olive oil and pumice.

2.2.2. In-process controls

Adequate growth of culture and cultural purity need to be checked. Presence of contaminating organisms may be detected by conventional sterility tests on harvests. Tests for pathogenic mycobacteria are carried out by injection of moist culture, taken prior to blending with adjuvant and diluted tenfold in saline, into two guinea-pigs, each receiving 1 ml. These are observed for 8 weeks, killed humanely, and examined for any abnormal lesions.

2.2.3. Final product batch test

i) Sterility

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in Chapter 1.1.9. The vaccine organism will not normally grow to a detectable level in conventional sterility tests.

ii) Identity

Identity of culture is performed by Ziehl–Neelsen staining and PCR (see Section B.1.4).

iii) Safety

These tests are normally performed in laboratory animals, although multidose tests in target animals would also be satisfactory. A typical laboratory animal test would be as follows. Each of two guinea-pigs is inoculated, subcutaneously, with an acceptable batch of vaccine at a fraction of the cattle dose previously determined to give a nodule but no overt necrosis at the injection site. Animals are observed for 8 weeks, killed humanely and examined for any abnormal lesions.

iv) Batch potency

As protection tests appear to be impractical, a test of sensitising ability may be used. This may then be related to bacterial content based on weight. A typical test would be as follows: guinea-pigs are sensitised by intramuscular injection of 0.5 ml of a 100-fold dilution in liquid paraffin of the vaccine under test. Skin tests are performed 6 weeks after sensitisation using intradermal inoculations of 0.2 ml of at least three serial dilutions of an *MAP* antigen, such as johnin PPD, the dilutions being chosen to give expected skin reactions of from 8 mm to 25 mm diameter. Each guinea-pig receives several dilutions per flank, their distribution being chosen by a Latin square design. After 24–48 hours, skin reactions are measured. A reference preparation for tests of this type has not yet been fully established. Avian tuberculin PPD of known international unitage may be used as a skin test antigen in tests of this type to ensure that the vaccine is capable of producing adequate sensitisation (corresponding to the vaccination).

2.3. Requirements for regulatory approval

2.3.1. Manufacturing process

A preservative is normally included for vaccine in multidose containers.

2.3.2. Safety requirements

i) Target and non-target animal safety

See Section C1.2.2.3.

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

~~Live vaccines are not available. See Chapter 1.1.8.~~

iii) Precautions (hazards)

The vaccine causes some side-effects, nodule formation and sensitisation of animals to the tuberculin test (Gwozdz *et al.*, 2000). In humans, accidental injection of vaccine has resulted in chronic inflammatory reactions requiring surgical treatment.

2.3.3. Efficacy requirements

The vaccine should be used as part of a control programme and will not on its own provide complete protection against disease caused by *MAP*.

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

No DIVA vaccine yet available

2.3.5. Duration of immunity

After vaccination at the age of 14–30 days, the vaccination effect is expressed as the reduction in the rate of excretors among vaccinated animals as compared with nonvaccinated bovines.

C2. Johnin

1. Background

Johnin PPD is a preparation of the heat-treated products of growth and lysis of *MAP*. Avian tuberculin PPD is a preparation of heat-treated products of growth and lysis of *M. avium* subsp. *avium* D4ER or TB 56. Details of avian tuberculin PPD have been incorporated into Chapter 3.1.13 *Mammalian tuberculosis (infection with Mycobacterium tuberculosis complex)*. These two preparations are used, by intradermal injection, to reveal DTH as a means of identifying animals infected or sensitised with *MAP*.

Guidelines for the production of veterinary biologicals 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 Johnin

2.1. Characteristics of the seed

2.1.1. Quality criteria

Cultures should be checked by staining smears for the presence of contaminating organisms.

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 at 5-day intervals, with 0.01 mg of the preparation under test in a volume of 0.1 ml. Each guinea-pig, together with each of 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 Johnin. The reactions of the two groups of guinea-pigs should not be significantly different when measured 24–48 hours later.

2.1.2. Validation as a strain

Strains of *MAP* used to prepare seed cultures should be identified by biotyping or genetic tests. They should be shown to be free from contaminating organisms.

2.2. Method of manufacture

2.2.1. Procedure

Johnin for skin test diagnosis is a PPD prepared from one or more strains of *MAP*. It may be prepared by the following method.

MAP strains are grown as a pellicle on liquid Reid's medium. Production cultures are usually inoculated from liquid seeding cultures rather than directly from seed on solid medium (Reid's synthetic medium). Production cultures are incubated at 37°C for 10 weeks.

At the end of the incubation period, the culture medium has a pH of about 5 and little or no johnin will be obtained unless the pH is raised, using sodium hydroxide, to about 7.3 before steaming. After thorough mixing, the cultures are free steamed for 3 hours. The bulk of the killed organisms is removed by coarse filtration and the filtrate is clarified by further filtration. Protein in the filtrate is precipitated chemically with 40% trichloroacetic acid, washed and redissolved (alkaline solvent). The product is sterilised by filtration. An antimicrobial preservative that does not give rise to false-positive reactions, such as phenol (not more than 0.5% [w/v]), may be added. Glycerol (not more than 10% [w/v]) may be added as a stabiliser. The product is dispensed aseptically into sterile glass containers, which are then sealed.

2.2.2. In-process controls

After final filtration the sterility of each filtrate of the PPD solution is checked.

Sterile filtrates are tested for protein content by a Kjeldahl method (British Pharmacopoeia [Veterinary], 1985). The protein content is adjusted to give between 0.475 and 0.525 mg/ml of protein in the final product. The pH is adjusted to the range 6.5–7.5.

2.2.3. Final product batch test

i) Sterility

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in Chapter 1.1.9. The organism will not normally grow to a detectable level in conventional sterility tests.

ii) Identity

Identity of culture is performed by Ziehl–Neelsen staining and PCR (see Section B.1.4).

iii) Safety

Two guinea-pigs should each be injected subcutaneously with 0.5 ml of the johnin under test. No significant local or systemic lesions should be seen within 7 days (British Pharmacopoeia [Veterinary], 1985).

Tests on johnin for living mycobacteria may be performed either on the material immediately before it is dispensed into final containers or on samples taken from final containers themselves. A sample of at least 10 ml should be taken, and this should be injected intraperitoneally or subcutaneously into at least two guinea-pigs, dividing the volume to be tested equally between the guinea-pigs. It is desirable to take a larger sample, say 50 ml, and to concentrate any residual mycobacteria by centrifugation or membrane filtration. The guinea-pigs are observed for at least 42 days and post-mortem examinations are carried out. Any macroscopic lesions are examined microscopically and culturally.

iv) Batch potency

The potency of johnin is currently determined by chemical assay for protein using a Kjeldahl method. A PPD content of 0.5 ± 0.025 mg/ml of final product is recommended (British Pharmacopoeia [Veterinary], 1985).

2.3. Requirements for regulatory approval

For johnin, the phenol used is no more than 0.5% (w/v). The concentration of the preservative in the final product and its persistence through shelf life should be checked.

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NB: There are WOA Reference Laboratories for paratuberculosis
(please consult the WOA Web site at:

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

Please contact the WOA Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for paratuberculosis

NB: FIRST ADOPTED IN 1991. MOST RECENT UPDATES ADOPTED IN 2021.

Appendix 1

Paratuberculosis: Intended purpose of test: population freedom from infection

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>PCR</u> ☒ ☐ ☐ <u>Bovine, sheep, goats, alpacas, llamas</u>	<u>Feces and tissues</u> <u>IS900, F57 and HspX</u>	<u>Systematic review and meta-analysis using cultures as gold standard</u> <u>Pooled sensitivity 0.92 (95% CI 0.85–0.96)</u> <u>Pooled specificity 0.85 (95% CI 0.77–0.91)</u>	<u>Mixed populations, 43 studied from 24 scientific articles were considered in this meta-analysis</u>	<u>See reference</u>	<u>Rapidity, good sensitivity and specificity compared with cultural methods</u>	<u>More expansive than ELISA</u> <u>Necessity for specific equipment and well-trained personnel</u> <u>It could have poor sensitivity when applied to a population with unknown paratuberculosis status</u>	<u>Liu et al. (2025)</u>
<u>Culture</u> ☒ ☐ ☐ <u>Bovine, sheep, goats,</u>	<u>Feces</u>	<u>Review reporting sensitivity and specificity variable according to the status (affected, infectious, infected) of the animals</u> <u>Sensitivity is lower than specificity</u>	<u>Mixed populations. Six evaluations for the bovine population, one for goats and sheep, respectively</u>	<u>See reference</u>	<u>No false positives if coupled with other confirmatory test.</u> <u>Semiquantitative outputs.</u>	<u>Presence of fastidious field isolates unable to grow</u> <u>The decontamination process reduces the analytical sensitivity, but the contaminant flora can impact as well</u> <u>A long time is required for the first isolation</u>	<u>Nielsen & Toft (2008)</u>
<u>AGID</u> ☒ ☒ ☐ <u>Different species</u>	<u>serum</u>	<u>Se: 13.8% for sheep and 39.5% for goats</u> <u>Sp: 100%</u>		<u>See reference</u>	<u>Possibility of working with non-mammalian animals or zoo animals.</u> <u>Speed</u> <u>Economically accessible.</u> <u>possibility of doing it in poorly equipped laboratories</u>	<u>Lower sensitivity than ELISAs</u>	<u>Gumber et al. (2006)</u>
<u>ELISA</u> ☒ ☒ ☐ <u>Cattle</u>	<u>Serum and milk</u>	<u>Se: between 54 and 100%</u> <u>Sp: between 47 and 94%</u> <u>Depending on, among other things, the kit used, the type of sampling, the individual prevalence, the test used as a reference, the type of analysis, the status of the animals</u>	<u>Targeted sampling, random sampling, prevalence stratification, repeated sampling, etc.</u>	<u>See reference</u>	<u>Speed, relatively good sensitivity and specificity compared with cultural methods. Economically accessible based on the sampling performed. Ease of sample collection</u>	<u>Se and Sp values vary widely depending on herd prevalence, infection status, and disease progression</u> <u>Commercial kits with local validations</u>	<u>Field et al. (2022)</u>

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
		(infected, sick), and the size of the herd					
ELISA ☒ ☒ ☐ Dairy cattle	Bulk milk tank	Se: Between 10 and 97% Sp: Between 95 and 100% Depending on, among other things, the kit used, the type of sampling, the individual prevalence, the test used as a reference, the type of analysis, the status of the animals (infected, sick), and the size of the herd	Targeted sampling, random sampling, prevalence stratification, repeated sampling, etc.	See reference	Speed, relatively good sensitivity and specificity compared with cultural methods Economically accessible based on the sampling performed Ease of sample collection	Se and Sp values vary widely depending on herd prevalence, infection status, and disease progression Commercial kits with local validations	Field <i>et al.</i> (2022)
ELISA ☒ ☒ ☐	Goats, sheep, deer	Goat: Se: between 82 and 100% (affected animals) 63 and 84% (infected animals) Sp: 92–100% Sheep: 36–85% (affected animals) 16–44% (infected animals) Sp: 95–99% Deer: Se: 83% (affected animals) Sp: 98–100%		See reference	Speed, relatively good sensitivity and specificity compared with cultural methods Economically accessible based on the sampling performed Ease of sample collection	Se and Sp values vary widely depending on herd prevalence, infection status, and disease progression Commercial kits with local validations Few systematic studies carried out	Nielsen & Toft (2008)
IFN- γ release assay ☒ ☐ ☐ bovine	Blood of individual animal	1 Reference) Longitudinal study evaluating the accuracy. The sensitivity and specificity varied according to the cut-off values selected. See reference for more details 2 Reference) Review reporting sensitivity and specificity for infectious animals. Sensitivity is	For the first reference, 235 animals divided in three groups: 235 animals divided in three groups: • 87 dairy cattle from three bovine tuberculosis officially free herds, where clinical cases of PTB had been reported • 61 beef cattle from a	See references	Early detection of infected animals. The first reference offers also some suggestions on the appropriateness of this method in the context of paratuberculosis	Poor diagnostic specificity. Necessity for well-trained personnel Technically demanding	Corneli <i>et al.</i> (2021) Nielsen & Toft (2008)

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
		<u>very variable but lower than specificity</u>	<u>bovine tuberculosis Officially Free herd without PTB cases; the herd had tested negative for serological tests in the last 4 years</u> <u>• 87 beef cattle from a bovine Tuberculosis positive herd with an ongoing outbreak when the study was performed</u>				

Appendix 2

Paratuberculosis:

Intended purpose of test: individual animal freedom from infection prior to movement

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>PCR</u> ☒ ☐ ☐ <u>Bovine, sheep, goats, alpacas, llamas</u>	<u>Feces and tissues</u> <u>IS900, F57 and HspX</u>	<u>Systematic review and meta-analysis using cultures as gold standard</u> <u>Pooled sensitivity 0.92 (95% CI 0.85–0.96)</u> <u>Pooled specificity 0.85 (95% CI 0.77–0.91)</u>	<u>Mixed populations. Forty-three studied from 24 scientific articles were considered in this meta-analysis</u>	<u>See reference</u>	<u>Rapidity, good sensitivity and specificity compared with cultural methods</u>	<u>Cost more elevated than ELISA</u> <u>Necessity for specific equipment and well-trained personnel</u> <u>It could have poor sensitivity when applied to a population with unknown paratuberculosis status</u>	<u>Liu <i>et al.</i> (2025)</u>
<u>Culture</u> ☒ ☐ ☐ <u>Bovine, sheep, goats</u>	<u>Feces</u>	<u>Review reporting sensitivity and specificity variable according to the status (affected, infectious, infected) of the animals. Sensitivity is lower than specificity</u>	<u>Mixed populations. Six evaluations for bovine population, one for goats and one for sheep, respectively</u>	<u>See reference</u>	<u>No false positive if coupled with other confirmatory test</u> <u>Semiquantitative outputs.</u>	<u>Presence of fastidious field isolates unable to grow</u> <u>The decontamination process reduces the analytical sensitivity, but the contaminant flora can impact as well</u> <u>A long time is required for the first isolation</u>	<u>Nielsen & Toft (2008)</u>
<u>AGID</u> ☒ ☐ ☐ <u>Different species</u>	<u>Blood of individual animal</u>	<u>Not recommended due to its low sensitivity in infected but not affected animals</u>		<u>See reference</u>	<u>Possibility of working with non-mammalian animals or zoo animals</u> <u>Speed</u> <u>Economically accessible</u> <u>Possibility of doing it in poorly equipped laboratories</u>	<u>Not recommended due to its low sensitivity</u>	<u>Gumber <i>et al.</i> (2006)</u>
<u>ELISA</u> ☒ ☒ ☐ <u>Different species</u>	<u>Serum and milk</u>	<u>Se: between 54 and 100%</u> <u>Sp: between 47 and 94%</u> <u>Depending on, among other things, the kit used, the type of sampling, the individual prevalence, the test used as a reference, the</u>		<u>See reference</u>	<u>Speed, relatively good sensitivity and specificity compared with cultural methods</u> <u>Economically accessible based on the sampling performed</u> <u>Ease of sample collection</u>	<u>Se and Sp values vary widely depending on herd prevalence, infection status, and disease progression</u> <u>Commercial kits with local validations.</u> <u>Few systematic studies carried out</u>	<u>Field <i>et al.</i> (2022)</u>

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
		<u>type of analysis, the status of the animals (infected, sick), and the size of the herd</u>					
<u>IFN-γ release assay</u> ☒ ☐ ☐ <u>bovine</u>	<u>Blood of individual animal</u>	<u>1 Reference) Longitudinal study evaluating the accuracy. The sensitivity and specificity varied according to the cut-off values selected. See reference for more details</u> <u>2 Reference) Review reporting sensitivity and specificity for infectious animals. Sensitivity is very variable but lower than specificity</u>	<u>For the first reference, 235 animals divided in three groups: 235 animals divided in three groups:</u> <u>• 87 dairy cattle from three bovine tuberculosis Officially Free herds, where clinical cases of PTB had been reported</u> <u>• 61 beef cattle from a bovine tuberculosis Officially Free herd without PTB cases; the herd had tested negative for serological tests in the last 4 years</u> <u>• 87 beef cattle from a bovine tuberculosis positive herd with an ongoing outbreak when the study was performed</u>	<u>See reference</u>	<u>Early detection of infected animals. The first reference offers also some suggestions on the appropriateness of this method in the context of paratuberculosis</u>	<u>Poor diagnostic specificity</u> <u>Necessity for well-trained personnel</u> <u>Technically demanding</u>	<u>Corneli <i>et al.</i> (2021)</u> <u>Nielsen & Toft (2008)</u>

Appendix 3

Paratuberculosis Intended purpose of test: contribute to eradication policies

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
PCR ☒ ☒ ☒ Bovine, sheep, goats, alpacas, llamas	Feces and tissues IS900, F57 and HspX	Systematic review and meta-analysis using cultures as gold standard Pooled sensitivity 0.92 (95% CI 0.85-0.96) Pooled specificity 0.85 (95% CI 0.77-0.91)	Mixed populations. Forty-three studied from 24 scientific articles were considered in this meta-analysis	See reference	Rapidity, good sensitivity and specificity compared with cultural methods	Cost more elevated than ELISA Necessity of specific equipment and well-trained personnel It could have poor sensitivity when applied to a population with unknown paratuberculosis status	Liu <i>et al.</i> (2025)
Culture ☒ ☐ ☐ Bovine, sheep, goats	Feces	Review reporting sensitivity and specificity variable according to the status (affected, infectious, infected) of the animals. Sensitivity is lower than specificity	Mixed populations, six evaluations for bovine population, one for goats and one for sheep, respectively	See reference	No false positive if coupled with other confirmatory test Semiquantitative outputs.	Presence of fastidious field isolates unable to grow The decontamination process reduces the analytical sensitivity, but the contaminant flora can impact as well A long time is required for the first isolation	Nielsen & Toft (2008)
Histopathology ☒ ☐ ☐ sheep	Tissues	Study comparing histopathology with liquid culture in TrekÔ system. Histopathology detected 80.9% of positive animals	104 sheep from flocks with a known history of MAP infection.	Not validated	Relatively inexpensive and not technically demanding	It requires very well-trained personnel for evaluation Accuracy unknown as the method was not employed in extensive studies and very often is used as ancillary test to confirm clinical cases Only post-mortem test	Hlokwe <i>et al.</i> (2025)
AGID ☒ ☐ ☐ Different species	Blood of individual animal	Not recommended due to its low sensitivity			Possibility of working with non-mammalian animals or zoo animals Speed Economically accessible. possibility of doing it in poorly equipped laboratories		Gumber <i>et al.</i> (2006)

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>ELISA</u> ☒ ☒ ☒ <u>Different species</u>	<u>Serum</u>	<u>Se: approx. between 54 and 100%</u> <u>Sp: between 47 and 94%</u> <u>Depending on, among other things, the kit used, the type of sampling, the individual prevalence, the test used as a reference, the type of analysis, the status of the animals (infected, sick), and the size of the herd</u>	<u>Targeted sampling, random sampling, prevalence stratification, repeated sampling, etc.</u>	<u>See reference</u>	<u>Speed, relatively good sensitivity and specificity compared with cultural methods</u> <u>Economically accessible based on the sampling performed</u> <u>Ease of sample collection</u>	<u>Se and Sp values vary widely depending on herd prevalence, infection status, and disease progression</u> <u>Commercial kits with local validations.</u>	<u>Field <i>et al.</i> (2022)</u>
<u>IFN-γ release assay</u> ☒ ☐ ☐ <u>bovine</u>	<u>Blood of individual animal</u>	<u>1 Reference) Longitudinal study evaluating the accuracy. The sensitivity and specificity varied according to the cut-off values selected. See reference for more details</u> <u>2 Reference) Review reporting sensitivity and specificity for infectious animals. Sensitivity is very variable but lower than specificity</u>	<u>For the first reference, 235 animals divided in three groups:</u> <u>• 87 dairy cattle from three bovine tuberculosis Officially Free herds, where clinical cases of PTB had been reported</u> <u>• 61 beef cattle from a bovine tuberculosis Officially Free herd without PTB cases; the herd had tested negative for serological tests in the last 4 years</u> <u>• 87 beef cattle from a bovine Tuberculosis positive herd with an ongoing outbreak when the study was performed</u>	<u>See references</u>	<u>Early detection of infected animals</u> <u>The first reference offers also some suggestions on the appropriateness of this method in the context of paratuberculosis</u>	<u>Poor diagnostic specificity</u> <u>Necessity of well-trained personnel</u> <u>Technically demanding</u>	<u>Corneli <i>et al.</i> (2021)</u> <u>Nielsen & Toft (2008)</u>
<u>DTH</u> ☒ ☐ ☐ <u>bovine</u>	<u>Blood of individual animal</u>	<u>Study comparing the specificity of DTH vs IFN-γ release assay</u>	<u>20 young stock (age 6–24 months) and 20 adult animals (>24 months) from 35 certified free herds</u> <u>537 young stock from</u>	<u>See the reference, but note that the study only focused on assay specificity</u>	<u>Specificity variable but high (93.5%) if a cut-off level >4 mm is chosen</u>	<u>Laborious to perform in the field</u> <u>Necessity for well-trained personnel</u> <u>Operators are at risk of being stung</u>	<u>Kalis <i>et al.</i> (2003)</u>

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
			<u>10, low prevalence (<5%) infected herds</u>				

Appendix 4

Paratuberculosis Intended purpose of test: confirmation of clinical cases

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>PCR</u> ☒ ☒ ☒ <u>Bovine, sheep, goats, alpacas, llamas</u>	<u>Feces and tissues</u> <u>IS900, F57 and HspX</u>	<u>Systematic review and meta-analysis using cultures as gold standard</u> <u>Pooled sensitivity 0.92 (95% CI 0.85-0.96)</u> <u>Pooled specificity 0.85 (95% CI 0.77-0.91)</u>	<u>Mixed populations. Forty-three studied from 24 scientific articles were considered in this meta-analysis</u>	<u>See reference</u>	<u>Rapidity, good sensitivity and specificity compared with cultural methods</u>	<u>Cost more elevated than ELISA</u> <u>Necessity for specific equipment and well-trained personnel</u> <u>It could have poor sensitivity when applied to a population with unknown paratuberculosis status</u>	<u>Liu <i>et al.</i> (2025)</u>
<u>Culture</u> ☒ ☒ ☒ <u>Bovine, sheep, goats</u>	<u>Feces</u>	<u>Review reporting sensitivity and specificity variable according to the status (affected, infectious, infected) of the animals. Sensitivity is lower than specificity</u>	<u>Mixed populations, six evaluations for bovine population, one for goats and one for sheep, respectively</u>	<u>See reference</u>	<u>No false positive if coupled with other confirmatory test. Semiquantitative outputs</u>	<u>Presence of fastidious field isolates unable to grow</u> <u>The decontamination process reduces the analytical sensitivity, but the contaminant flora can impact as well</u> <u>A long time is required for the first isolation</u>	<u>Nielsen & Toft (2008)</u>
<u>Faecal ZN staining</u> ☒ ☐ ☐ <u>Bovine</u>	<u>Faeces</u>	<u>Study analysing clinical case of paratuberculosis in dairy cows</u>	<u>Five dairy cows aged 2 (n = 2) or 3 (n = 3). The diagnosis included positive MAP antibody test and PCR positive faecal test.</u>	<u>Not validated</u>	<u>Rapid and cheap</u> <u>Useful for many animal species</u>	<u>Sensitivity and specificity in general very low</u>	<u>Cheng <i>et al.</i> (2020)</u>
<u>Histopathology</u> ☒ ☒ ☒ <u>Sheep, bovine</u>	<u>Tissues</u>	<u>First Study compared histopathology with liquid culture in TrekÔ system. Histopathology detected 80.9% of positive animals</u> <u>The second study analysed clinical case of paratuberculosis in dairy cows</u>	<u>First study: 104 sheep from flocks with a known history of MAP infection</u> <u>Second study: five dairy cows aged 2 (n = 2) or 3 (n = 3). The diagnosis included positive MAP antibody test and PCR positive faecal test</u>	<u>Not validated</u>	<u>Relatively cheap and not technically demanding</u>	<u>It requires very well-trained personnel for evaluation</u> <u>Accuracy unknown as the method was not employed in extensive studies and very often is used as ancillary test to confirm clinical cases</u> <u>Only post-mortem test</u>	<u>Hlokwe <i>et al.</i> (2025)</u> <u>Cheng <i>et al.</i> (2020)</u>

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
AGID ☑☑☐ <u>Different species</u>	<u>Serum</u>	<u>Se: 13.8% for sheep and 39.5% for goats</u> <u>Sp: 100%</u>		<u>See reference</u>	<u>Possibility of working with non-mammalian animals or zoo animals</u> <u>Speed</u> <u>Economically accessible.</u> <u>possibility of doing it in poorly equipped laboratories</u>	<u>Lower sensitivity than ELISAs</u>	<u>Gumber <i>et al.</i> (2006)</u>
ELISA ☑☑☑ <u>Cattle</u>	<u>Serum</u>	<u>Se: approx. between 50 and 100%</u> <u>Depending on, among other things, the kit used, the type of sampling, the individual prevalence, the test used as a reference, the type of analysis, the status of the animals (infected, sick), and the size of the herd</u>		<u>See reference</u>	<u>Speed, relatively good sensitivity and specificity compared with cultural method</u> <u>Economically accessible based on the sampling performed</u> <u>Ease of sample collection</u>	<u>Se and Sp values vary widely depending on herd prevalence, infection status, and disease progression</u> <u>Commercial kits with local validations</u> <u>Few systematic studies carried out</u>	<u>Field <i>et al.</i> (2022)</u>
ELISA ☑☑☑ <u>Different species</u>	<u>Serum</u>	<u>Goat.</u> <u>Se: 82–100% (affected animals)</u> <u>Sp: 92-100% (affected animals)</u> <u>Sheep.</u> <u>Se: 36–85% (affected animals)</u> <u>Sp: 95–99% (affected animals)</u> <u>Depending on, among other things, the kit used, the type of sampling, the individual prevalence, the test used as a reference, the type of analysis, the status of the animals (infected, sick), and the size of the herd</u>		<u>See reference</u>	<u>Speed, relatively good sensitivity and specificity compared with cultural methods</u> <u>Economically accessible based on the sampling performed</u> <u>Ease of sample collection</u>	<u>Se and Sp values vary widely depending on herd prevalence, infection status, and disease progression</u> <u>Commercial kits with local validations</u> <u>Few systematic studies carried out</u>	<u>Dimareli-Malli <i>et al.</i> (2004)</u>

Appendix 5

Paratuberculosis Intended purpose of test: prevalence of infection – surveillance

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>PCR</u> ☒ ☒ ☒ <u>Bovine, sheep, goats, alpacas, llamas</u>	<u>Feces and tissues</u> <u>IS900, F57 and HspX</u>	<u>Systematic review and meta-analysis using cultures as gold standard</u> <u>Pooled sensitivity 0.92 (95% CI 0.85-0.96)</u> <u>Pooled specificity 0.85 (95% CI 0.77-0.91)</u>	<u>Mixed populations. Forty-three studied from 24 scientific articles were considered in this meta-analysis</u>	<u>See reference</u>	<u>Rapidity, good sensitivity and specificity compared with cultural methods</u>	<u>Cost more elevated than ELISA</u> <u>Necessity for specific equipment and well-trained personnel</u> <u>It could have poor sensitivity when applied to a population with unknown paratuberculosis status</u>	<u>Liu <i>et al.</i> (2025)</u>
<u>Culture</u> ☒ ☒ ☐ <u>Bovine, sheep, goats</u>	<u>Feces</u>	<u>Review reporting sensitivity and specificity variable according to the status (affected, infectious, infected) of the animals. Sensitivity is lower than specificity</u>	<u>Mixed populations, six evaluations for bovine population, one for goats and one for sheep, respectively</u>	<u>See reference</u>	<u>No false positive if coupled with other confirmatory tests</u> <u>Semiquantitative outputs</u>	<u>Presence of fastidious field isolates unable to growth</u> <u>The decontamination process reduces the analytical sensitivity, but the contaminant flora can impact as well</u> <u>A long time is required for the first isolation</u>	<u>Nielsen & Toft (2008)</u>
<u>Faecal ZN staining</u> ☒ ☐ ☐ <u>Bovine</u>	<u>Faeces</u>	<u>Study analysing clinical case of paratuberculosis in dairy cows</u>	<u>Five dairy cows aged 2 (n = 2) or 3 (n = 3). The diagnosis included positive MAP antibody test and PCR positive faecal test.</u>	<u>Not validated</u>	<u>Rapid and inexpensive</u> <u>Useful for many animal species</u>	<u>Sensitivity and specificity in general very low</u>	<u>Cheng Z <i>et al.</i> (2020).</u>
<u>Histopathology</u> ☒ ☐ ☐ <u>Sheep, bovine</u>	<u>Tissues</u>	<u>First study compared histopathology with liquid culture in TrekÔ system. Histopathology detected 80.9% of positive animals</u> <u>The second study analysed clinical case of paratuberculosis in dairy cows</u>	<u>First study: 104 sheep from flocks with a known history of MAP infection</u> <u>Second study: five dairy cows aged 2 (n = 2) or 3 (n = 3). The diagnosis included positive MAP antibody test and PCR positive faecal test</u>	<u>Not validated</u>	<u>Relatively inexpensive and not technically demanding</u>	<u>It requires very well-trained personnel for evaluation</u> <u>Accuracy unknown as the method was not employed in extensive studies and very often is used as ancillary test to confirm clinical cases</u> <u>Only post-mortem test</u>	<u>Hlokwe <i>et al.</i> (2025)</u> <u>Cheng <i>et al.</i> (2020)</u>

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
AGID ☒ ☒ ☐ <u>Different species</u>	<u>Blood of individual animal</u>			<u>Not validated</u>	<u>Possibility of working with non-mammalian animals or zoo animals.</u> <u>Speed</u> <u>Economically accessible.</u> <u>possibility of doing it in poorly equipped laboratories</u>		<u>Gumber <i>et al.</i> (2006)</u>
ELISA ☒ ☒ ☒ <u>Different species</u>	<u>Serum</u>	<u>Se: between 54 and 100%</u> <u>Sp: between 47 and 94%</u> <u>Depending on, among other things, the kit used, the type of sampling, the individual prevalence, the test used as a reference, the type of analysis, the status of the animals (infected, sick), and the size of the herd</u>		<u>See reference</u>	<u>Speed, relatively good sensitivity and specificity compared with cultural methods</u> <u>Economically accessible based on the sampling performed</u> <u>Ease of sample collection</u>	<u>Se and Sp values vary widely depending on herd prevalence, infection status, and disease progression</u> <u>Commercial kits with local validations</u> <u>Few systematic studies carried out</u>	<u>Nielsen & Toft (2008)</u>
ELISA ☒ ☒ ☒ <u>Dairy cattle</u>	<u>Bulk milk tank</u>	<u>Se: Between 10 and 97%</u> <u>Sp: Between 95 and 100%</u> <u>Depending on, among other things, the kit used, the type of sampling, the individual prevalence, the test used as a reference, the type of analysis, the status of the animals (infected, sick), and the size of the herd</u>	<u>Targeted sampling, random sampling, prevalence stratification, repeated sampling, etc.</u>	<u>See reference</u>	<u>Speed, relatively good sensitivity and specificity compared with cultural methods</u> <u>Economically accessible based on the sampling performed</u> <u>Ease of sample collection</u>	<u>Se and Sp values vary widely depending on herd prevalence, infection status, and disease progression</u> <u>Commercial kits with local validations</u>	<u>Field <i>et al.</i> (2022)</u>

Appendix 6

Paratuberculosis

Intended purpose of test: immune status in individual animals or populations post-vaccination

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
AGID ☒ ☒ ☐	<u>Serum</u>	<u>Data not available</u>	<u>Data not available</u>	<u>Data not available</u>	<u>Theoretically, this test could be applied at the herd level to assess if the animals have mounted an immunological response vs MAP, used as a proxy to control for successful vaccination</u>	<u>- Loss of differentiation between infected and vaccinated animals (DIVA). Not useful for assessing immune response at the individual level</u>	
<u>Serum ELISA</u> ☒ ☒ ☐ <u>bovine, goats</u>	<u>Serum bovines</u> <u>Milk goats</u>	<u>Data not available</u>	<u>Seven dairy herds, 1288 animals for bovines; 49 dairy herds for goats</u>	<u>See publications</u>	<u>Theoretically, this test could be applied at the herd level to assess the apparent success of vaccination. The proportion of positive animals is significantly higher in the vaccinated than in the non-vaccinated animals</u>	<u>Loss of differentiation between infected and vaccinated animals (DIVA). The ELISA response decreases over time after boosting. Not useful for assessing immune response at the individual level</u>	<u>Corbiere <i>et al.</i> (2023) for bovines</u> <u>Luttikholt <i>et al.</i> (2019) for goats</u>
<u>IFN-γ release assay</u> ☒ ☒ ☐	<u>Serum</u>	<u>Data not available</u>	<u>Data not available</u>	<u>Data not available</u>	<u>Theoretically, this test could be applied at the herd level to assess the apparent success of vaccination</u>	<u>- Interference with the immune tests used in bovine tuberculosis eradication programmes</u> <u>Loss of differentiation between infected and vaccinated animals (DIVA)</u>	
<u>DTH</u> ☒ ☒ ☐ <u>Bovine</u>	<u>Serum</u>	<u>Data not available</u>	<u>Seve, dairy and beef herds</u>	<u>See publication</u>	<u>Theoretically, this test could be applied at the herd level to assess the apparent success of vaccination</u>	<u>- Interference with the immune tests used in bovine tuberculosis eradication programmes (vaccinated animals showed seventeen times more reactions than the non-vaccinated in the single bovine intradermal tuberculin test, and four times more in the comparative intradermal test)</u> <u>Loss of differentiation between infected and vaccinated animals (DIVA)</u>	<u>Garrido <i>et al.</i> (2013)</u>

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CHAPTER 3.3.3.

AVIAN INFECTIOUS LARYNGOTRACHEITIS

SUMMARY

Description of the disease: Avian infectious laryngotracheitis (ILT) is a respiratory disease caused by gallid alphaherpesvirus 1. It is principally a disease of chickens, although it can also affect pheasants, partridges and peafowl. The clinical signs and observed pathology reactions may vary from extremely severe, with some birds dying from asphyxiation, to very mild, indistinguishable from other mild respiratory diseases of chickens. The principal lesion is tracheitis. In infected birds, the virus can become latent and be re-excreted at a later date without clinical signs.

Laboratory diagnosis depends on isolation of the virus, and demonstration of the presence of the virus, viral antigens or DNA. Histopathological examination of the trachea for characteristic intranuclear inclusions and syncytial cell formations are of diagnostic value. Many of the diagnostic methods described in this chapter have not undergone formal validation to modern standards, but are accepted through widespread use over a prolonged period ("historical validation").

Detection of the agent: Virus isolation may be done by inoculation of suspected material on to the dropped chorioallantoic membrane of embryonated hens' eggs, or into avian embryonic cell cultures. These methods are time-consuming but remain useful. Rapid methods include immunofluorescence on tracheal exudate or frozen sections, and an enzyme-linked immunosorbent assay (ELISA) to demonstrate viral antigen in mucosal scrapings. Polymerase chain reaction (PCR) methodology has been shown to be more sensitive than virus isolation for the examination of clinical material and is now widely used. Both conventional and real-time PCR are used to detect ILTV DNA. Real-time PCR provides a relative quantification of viral genomes per sample, which can indicate the stage of infection and level of virus transmission in the flock. Currently, histopathology examination of tissues and real-time PCR are the most common pair of rapid assays used for the detection of ILTV infection. Virus characterisation and differentiation of vaccine and wild-type viruses are possible using PCR followed by restriction fragment length polymorphism or sequencing.

Serological tests: Antibodies to ILT virus can be detected by virus neutralisation (VN) tests conducted in eggs or in cell cultures, or by AGID reactions, indirect immunofluorescence, or ELISA. The latter is preferred for screening flocks.

Requirements for vaccines: Vaccines against ILT are usually prepared from attenuated live virus. Those available at present afford some degree of protection, but are not ideal. A number of recombinant vaccines are also available commercially with various degrees of protective efficacy.

A. INTRODUCTION

Avian infectious laryngotracheitis (ILT) is a respiratory disease of chickens caused by *gallid alphaherpesvirus 1* (*Iltoavirus gallidalpha1*). It can also affect pheasants, partridges and peafowl. In the virulent form, the history, clinical

signs and very severe tracheal lesions are highly characteristic of the disease, but the mild form may be indistinguishable from other agents causing mild respiratory diseases. Laboratory diagnosis depends on the demonstration of the presence of the virus or viral components or specific antibodies in the serum.

Clinically, the disease may appear in three forms, namely peracute, subacute, and chronic or mild. In the peracute form, onset of disease is sudden with a rapid spread. The morbidity is high and mortality may exceed 50%. Some birds may die in good body condition before the appearance of signs, which are characteristic and comprise difficulty in breathing with extension of the neck and gasping in an attempt to inhale. There is also gurgling, rattling and coughing when birds try to expel obstructions in the trachea. Conjunctivitis may also be observed. Clots of blood may be coughed up and can be found on the floor and walls of the house. Post-mortem changes are confined to the upper respiratory tract and are also characteristic, consisting of haemorrhagic tracheitis with blood clots, mucoid rhinitis, and blood-stained mucus along the length of the trachea.

In the subacute form, the onset of illness is slower and respiratory signs may extend over some days before deaths are seen. The morbidity is high but the mortality is lower than in the peracute form, between 10% and 30%. Post-mortem findings are less severe and consist of mucoid exudate with or without blood in the trachea. Yellow caseous diphtheritic membranes may be found adherent to the larynx and upper tracheal mucosa.

Chronic or mild ILT may be seen among survivors of either of the above forms of the disease, although some outbreaks themselves may be entirely mild. Incidence of chronic ILT within a flock may be only 1–2%. Signs include coughing, nasal, ocular and oral discharge, and reduced egg production.

Differential diagnosis of ILT from other respiratory diseases, especially Newcastle disease, which may also cause severe tracheitis, is important. Also, diphtheritic oral lesions should be differentiated from the wet form of fowlpox.

Infection is acquired via the upper respiratory tract and transmission occurs most readily from acutely infected birds, but clinically inapparent infection can persist for long periods with intermittent re-excretion of the virus, and these recovered carrier birds are also a potential means of transmission of the disease (Hughes *et al.*, 1987). Given that transmission of ILT takes place by close contact, transmission is slower in cage houses than where birds are loose-housed, and the path of infection through a cage house may be apparent. Recent work has confirmed considerable variation among ILT virus (ILTV) strains in their tropism for trachea or conjunctiva and those with affinity for the latter site can severely affect weight gain (Kirkpatrick *et al.*, 2006a). There is increasing evidence that highly virulent and transmissible strains can emerge as a result of recombination between two or more different strains (Lee *et al.*, 2015).

There is no known risk of human infection with ILTV. Biocontainment measures should be 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*.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of avian infectious laryngotracheitis 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	□□□	□□□	□□□	☑☑□	□□□	□□□
Immuno-fluorescence for antigen	□□□	□□□	□□□	☑☑□	□□□	□□□
ELISA – antigen detection	□□□	☑☑□	□□□	☑☑☑	□□□	□□□
PCR	☑□□	☑☑☑	☑☑□	☑☑☑	☑☑☑	□□□

Histopathology	□□□	□□□	□□□	☑☑□	□□□	□□□
Detection of immune response						
VN	☑□□	□□□	☑□□	□□□	□□□	☑☑□
ELISA – antibody detection	☑☑□	☑□□	☑☑☑	☑□□	☑☑☑	☑☑☑

Key: ☑☑☑ = recommended for this purpose; ☑☑□ = recommended but has limitations;

☑□□ = suitable in very limited circumstances; □□□ = not appropriate for this purpose.

ELISA = Enzyme-linked immunosorbent assay; PCR = polymerase chain reaction; VN = virus neutralisation.

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

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

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

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

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

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

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

1. Detection of the agent

The virus may be isolated in chicken embryo liver, chicken embryo kidney or in chicken kidney cell cultures. Of these, monolayers of chicken embryo liver cells have been found to be the most sensitive (Hughes & Jones, 1988). The virus can also be grown on the dropped chorioallantoic membrane (CAM) of 10- to 12-day-old specific pathogen free (SPF) embryonated chicken eggs (Jordan, 1964).

The causative herpesvirus may be demonstrated directly in tracheal exudate by electron microscopy (Van Kammen & Spadbrow, 1976). Viral antigens may be detected by immunofluorescence (Braune & Gentry, 1985; Wilks & Kogan, 1979), or enzyme-linked immunosorbent assay (ELISA), using tracheal mucosal scrapings (York & Fahey, 1988). Histopathological examination of the trachea for typical herpesvirus intranuclear inclusions and syncytial cell formation may also be helpful (Pirozok *et al.*, 1957). Both conventional and real-time polymerase chain reaction (PCR) are used to detect ILTV nucleic acid. An additional advantage of real-time PCR is that it provides a relative quantification of viral genomes per sample, which can indicate the stage of infection and level of virus transmission in the flock. Conventional PCR is also useful in the diagnosis of ILTV because amplification products generated by conventional PCR can be sequenced for further virus genotyping (Menendez *et al.*, 2014).

1.1. Virus isolation

When samples are taken from live birds for virus isolation, tracheal swabs are superior to oropharyngeal or conjunctival swabs. These are placed in transport medium containing antibiotics. When selecting material for virus isolation from chronic outbreaks, it is more productive to cull a bird in the early stages of the infection, rather than to attempt to isolate virus from a bird that has died of asphyxiation after a long illness. The quality of sample is further improved if the bird is killed by barbiturate or other injection rather than by cervical dislocation. The whole head and neck from dead birds may be submitted, or only the trachea and larynx after their removal with minimal contamination. Tracheas should be transported in antibiotic broth for virus isolation, but wrapped in moist tissue paper if destined for electron microscopy. Any prolonged storage of infected tissues should be at –70°C or below to minimise loss of virus titre. Repeated freezing and thawing must be avoided as this reduces virus infectivity.

Exudate and epithelial cells are scraped from the tracheas, diluted approximately 1/5 in nutrient broth containing penicillin and streptomycin, and agitated vigorously. The resulting suspension is centrifuged at low speed to remove debris, and 0.1 ml of the supernatant fluid is inoculated on to the dropped CAM of at least three embryonated chicken eggs of 10–12 days' incubation. The eggs are sealed with paraffin wax and incubated at 37°C for up to 7 days. They are candled daily and the CAMs of dead embryos or of those surviving for 7 days are examined for typical pocks. Alternatively, at least two confluent chick embryo liver or chicken embryo kidney cell monolayers, with their medium removed, are inoculated and allowed to adsorb for 1–2 hours. Cultures are overlaid with fresh medium, incubated for up to 7 days and examined daily under the microscope for evidence of a typical syncytial cell cytopathic effect (CPE).

In each instance, up to three passages of material may be necessary before a specimen is considered to be negative. A virus isolate can be confirmed as ILTV by a neutralisation test in eggs or cell culture using hyperimmune antiserum to ILTV. Alternatively, virus particles may be identified rapidly in cell culture fluid or in pocks on CAMs by electron microscopy, viral antigens by immunofluorescence in acetone-fixed ILT-virus-infected cell cultures or in frozen sections of CAM and viral nucleic acid by PCR.

1.2. Immunofluorescence

In immunofluorescence tests for viral antigens, epithelial cell scrapings from the trachea are smeared on to a glass slide. Alternatively, 5 µm thick cryostat sections of trachea, snap-frozen in liquid nitrogen may be used. The preparations are fixed in acetone at room temperature for 10 minutes. These can be stained directly by applying chicken anti-ILTV immunoglobulin labelled with fluorescein isothiocyanate (FITC) for 1 hour, followed by rinsing for 15 minutes in a bath of phosphate-buffered saline (PBS), pH 7.2, agitated with a magnetic stirrer. Otherwise, they can be stained indirectly by applying an appropriate dilution of chicken anti-ILT serum for 1 hour. The slide is rinsed thoroughly with PBS for 15 minutes as above, and an FITC-labelled anti-chicken immunoglobulin is applied for 30 minutes. After a final rinse, cover-slips are applied over non-fade mountant. The preparations are examined for specific intranuclear fluorescence in the epithelial cells using a microscope with epifluorescent ultraviolet illumination. Suitable controls include the use of known uninfected specimens and, for the indirect method, the application of nonimmune chicken serum. Particular care should be taken in the reading of indirect immunofluorescence preparations, as endogenous chicken IgG in the trachea may cause unwanted attachment of FITC-labelled anti-chicken IgG.

1.3. Enzyme-linked immunosorbent assay

A number of antigen detection ELISA kits are available commercially, and the assay procedure may vary depending on the kit; the manufacturer's instructions should always be followed. When the monoclonal antibody (MAb) ELISA is used for detecting viral antigens (McNulty *et al.*, 1985), tracheal exudate is mixed with an equal volume of PBS containing 1% (v/v) of a nonionic, non-denaturing detergent, then vortexed for 30 seconds and centrifuged at 10,000 *g* for 1 minute. The supernatant fluid is dropped in 50 µl volumes in wells of microtitre plates previously coated with rabbit IgG against ILTV, diluted 1/200 in 0.05 M carbonate/bicarbonate buffer, pH 9.0, and incubated for 1 hour. Next, 50 µl of MAb against major glycoproteins of ILTV, diluted 1/50 in PBS, is added to each well, followed by 50 µl of a 1/1000 dilution of affinity-purified goat anti-mouse IgG conjugated to horseradish peroxidase. The chromogen/substrate, 5-aminosalicylic acid (6.5 mM) with hydrogen peroxide (to a final concentration of 0.0005%), is added to the wells in 100 µl volumes. After 30 minutes, the plates are read at 450 nm on a spectrophotometer and the absorbance reading for each well is corrected by subtracting the reading obtained for wells containing diluting buffer instead of tracheal exudate. The positive/negative cut-off point is taken as the mean absorbance value for several negative (i.e. tracheal material without ILTV) samples plus 3 standard deviations.

1.4. Histopathology

Birds selected for post-mortem examination should be in the acute phase of disease. Euthanasia should be by intravenous injection of barbiturates or exposure halothane, to avoid damaging the trachea. Tracheas for histopathological examination should be placed in 10% neutral buffered formalin or Bouin fixative (preferable for detection of intranuclear inclusion bodies) immediately after removal from the birds and, after fixation, embedded in paraffin wax. Eyelid and lung are sometimes examined. Intranuclear inclusions may be seen in the epithelial cells of the trachea after staining with haematoxylin and eosin. Syncytial cell formations are often present within the exudates and commonly contain intranuclear inclusion bodies. Inclusion bodies are the classical Cowdry type A inclusions of herpesviruses, but they may be present for only 3–5 days after infection. In severe cases where most infected cells have detached from the tracheal lining, inclusions may be seen in intact cells among the cellular debris in the lumen of the trachea. Longitudinal rather than transverse sections of trachea permit examination of the whole length of the organ.

1.5. Molecular methods

~~Several molecular methods for identifying ILTV DNA in clinical samples have been reported, but the PCR has proved the most useful. Using a nested PCR, Humbert *et al.* (2002) showed that ILTV DNA could be detected in formalin fixed, paraffin embedded tissues independently of the presence of syncytial cells, intranuclear inclusions or both.~~

~~PCR has been found to be more sensitive than virus isolation for clinical samples, especially when other contaminant viruses such as adenoviruses are present (Williams *et al.*, 1994). Alexander & Nagy (1997) found that during the middle to the end of the infection phase, PCR and virus isolation were similar in sensitivity, but PCR was superior in the recovery phase.~~

~~The combination of PCR with restriction fragment length polymorphism (RFLP) analysis of single and multiple viral genes and genome regions has enabled the characterisation of different strains within a country or region (Chang *et al.*, 1997). Several reports have shown that while some field strains are~~

closely related to and likely to be derived from vaccine viruses, others are true 'wild types' (Ojkie *et al.*, 2006). Genes commonly examined by different authors include ICP4, TK (thymidine kinase), glycoprotein G (gG), glycoprotein E (gE) and UL47. Oldoni & Garcia (2007) used 36 restriction enzymes, while others have used as few as four.

There is no molecular test that is universally recognised for strain identification of ILTVs, but a protocol has been provided below in Section B.1.5.1.iv *Restriction fragment length polymorphism* that has been used in a number of poultry diagnostic laboratories for differentiation of vaccines and field strains and epidemiological investigations. A good history of vaccination in the flock may assist with interpretation of results although it should be noted that field strain infection can occur in vaccinated birds. Vaccine strains can also occasionally be isolated from unvaccinated birds,

1.5.1. Test procedures

i) Conventional PCR

In a typical PCR protocol for ILTV, viral DNA is extracted from clinical samples (swabs, tissues), chorioallantoic membrane plaques, cell culture supernatants or vaccines using DNA extraction kits. Both commercial extraction kits and in-house extraction methods are used in diagnostic laboratories, but the quality and quantity of extracted nucleic acid may vary from one procedure to another. Manual extraction is practiced in many laboratories and could be highly efficient although nowadays many advanced laboratories make use of automated extraction and liquid handling to set up the PCR reactions.

~~Primers used can be obtained from previously published work or designed using ILTV sequences on the Genbank international database.~~

~~The following protocols is—are routinely used in a number of veterinary diagnostic laboratories for the detection of ILTV in clinical specimens and has potential for the. The advantage of these PCRs is that the PCR amplicons can be used immediately after PCR for the preliminary typing of the virus by restriction fragment length polymorphism (Kirkpatrick *et al.*, 2006b) or nucleotide sequencing of the PCR amplicons (Chacon & Ferreira, 2009). Full details of this PCR are provided in Kirkpatrick *et al.* (2006b).~~

Target gene	Primer (5'–3')	Concentration	Cycling parameters
Method 1: Kirkpatrick <i>et al.</i> (2006b); amplicon size: 2.24 kbp			
Thymidine kinase (TK)	TK forward: CTG-GGC-TAA-ATC-ATC-CAA-GAC-ATC-A TK reverse: GCT-CTC-TCG-AGT-AAG-AAT-GAG-TAC-A	250 nM 250 nM	One cycle of 94°C/3 min; 35 cycles of: 94°C/15 sec, 60°C/45 sec and 72°C/150 sec
Method 2: Chacon & Ferreira (2009); amplicon size: 688 bp			
Infected cell protein 4 gene (ICP4)	ICP4-1F: ACT-GAT-AGC-TTT-TCG-TAC-AGC-ACG ICP4-1R: CAT-CGG-GAC-ATT-CTC-CAG-GTA-GCA	500 nM 500 nM	One cycle of 94°C/3 min; 35 cycles of: 94°C/1 min, 62°C/1 min and 72°C/1.5 min; One cycles of 72°C/10 min

~~In brief, an approximately 2.24 kbp region of the ILTV thymidine kinase gene is amplified using a pair of forward (5' CTG GGC TAA ATC ATC CAA GAC ATC A 3') and reverse (5' GCT CTC TCG AGT AAG AAT GAG TAC A 3') primers and The resultant amplicons are separated by electrophoresis through 0.8% agarose gels, stained with an appropriate nucleic acid stain and exposed to UV light for visualisation. The 50 µl amplification reaction contains 200 µM each of dATP, dCTP, dGTP and dTTP, 1 mM MgCl₂, 250 µM each of the primers, 1 µl (2.5 units) Taq DNA polymerase, 5 µl of 10 × Taq DNA polymerase buffer and 5 µl extracted DNA as template. The reaction mixture should be incubated at 94°C for 3 minutes, then subjected to 35 cycles of 94°C for 15 seconds, 60°C for 45 seconds and 72°C for 150 seconds, and finally incubated at 72°C for 3 minutes. In each series of PCRs, a control tube containing sterile distilled H₂O, instead of extracted DNA template, should be included as a negative control.~~

~~Another protocol that has been used in veterinary diagnostic laboratories around the world for the detection and preliminary typing of the virus involves the amplification of two regions of the ICP4 gene. Primers ICP4 1F (5' ACT GAT AGC TTT TCG TAC AGC ACG 3') and ICP4 1R (5' CAT CGG GAC ATT CTC CAG GTA GCA 3') amplify a 688 bp fragment at~~

position 181 to 869¹; ICP4 2F (5'-CTT CAG ACT CCA GCT CAT CTG 3') and ICP4 2R (5'-AGT CAT GCG TCT ATG GCG TTG AC 3') amplify a 635 bp fragment at position 3804 to 4440². Full details of these PCRs are provided by Chacon & Ferreira (2009).

The real time PCR protocol described by Mahmoudian *et al.* (2011) can also be used as a conventional PCR with the final products can be examined by electrophoresis through 2% agarose gels.

ii) Real-time PCR

Real-time PCR assays have been described for ILTV including those described by (Creelan *et al.* (2006), Callison *et al.* (2007) and Mahmoudian *et al.* (2011). These have the advantage that they Real-time PCR assays can be conducted in less than 2 hours and therefore provide a very rapid method of ILT diagnosis in comparison with traditional virus isolation, or even the standard PCR followed by gel electrophoresis. The following protocol can be used in some diagnostic laboratories for the rapid detection of ILTV in clinical specimens.

<u>Target gene</u>	<u>Primer/probe (5'-3')</u>	<u>Concentration</u>	<u>Cycling parameters</u>
<u>Callison <i>et al.</i> (2007)</u>			
<u>Glycoprotein C (gC)</u>	ILTVgCU771 (CCT-TGC-GTT-TGA-ATT-TTT-CTG-T) ILTVgCL873 (TTC-GTG-GGT-TAG-AGG-TCT-GT) probe817 (FAM-CAG-CTC-GGT-GAC-CCC-ATT-CTA-BHQ1)	500 nM 500 nM 100 nM	One cycle of 50°C/2min and 95°C/15 min; 40 cycles of 94°C/15 sec and 60°C/1 min

Full details of the methodology are provided in Callison *et al.* (2007). In brief, two oligonucleotide primers, ILTVgCU771 (5'-CCT-TGC-GTT-TGA-ATT-TTT-CTG-T-3') and ILTVgCL873 (5'-TTC-GTG-GGT-TAG-AGG-TCT-GT-3'), are used to amplify a 103 bp product from the ILTV gC gene. A fluorescent labelled probe, ILTV probe817 (5'-FAM-CAG-CTC-GGT-GAC-CCC-ATT-CTA-BHQ1-3'), is also used. The reaction is conducted with appropriate PCR kits, for example, using 25 µl total volume containing 10 µl of the 2× master mix, 0.5 µM of forward and reverse primers, 0.1 µM of the probe, 1 µl of HK-UNG, and 5 µl of DNA template. Incubation and data acquisition are performed using a real time thermocycler although PCR protocols will need to be optimised for the machine used. Reactions are incubated for 2 minutes at 50°C, 15 minutes at 95°C, then 40 cycles of 15 seconds at 94°C and 1 minute at 60°C. For each assay, the threshold cycle number (CT value) is the PCR cycle number at which the fluorescence of the reaction exceeds 30 units of fluorescence.

iii) Nucleotide sequence analysis

A number of ILTV genes can be targeted for PCR followed by nucleotide sequence analysis of the resultant amplicons for strain identification purposes, for example, ICP4 may be amplified by PCR using the primers described by Chacon & Ferreira, (2009) and the resultant amplicons purified using a disposable mini column method and submitted to bi-directional DNA sequencing using the PCR primers as sequencing primers. Various software programs including clustal W may be used for analysis of the sequences and comparison with the existing sequences in GenBank. It should be noted that sequence analysis of multiple genes may be required for proper identification of the ILTV strains.

Whole genome sequencing has also been reported in several publications as a means of fully characterising the viral strains, but, at least currently, equipment, reagents and expertise are available only in a limited number of laboratories and therefore this technique has not been discussed here.

1 — Genbank accession number NC 006623
2 — Genbank accession number NC 006623

iv) Restriction fragment length polymorphism (RFLP)

The combination of PCR and RFLP has enabled field strains of ILTV to be distinguished from vaccine strains (Creelan *et al.*, 2006; Han & Sim, 2001; Kirkpatrick *et al.*, 2006b; Ojkic *et al.*, 2006; Oldoni & Garcia, 2007). A range of restriction endonucleases (RE) have been described for RFLP analysis of ILTV PCR products and several genes have been targeted for digestion. They include ICP4, TK, UL15, UL47 glycoprotein G and ORF-BTK genes. Detailed description of the methodology can be found in Creelan *et al.* (2006), Han & Sim (2001), Kirkpatrick *et al.* (2006b), Ojkic *et al.* (2006) and Oldoni & Garcia (2007). A brief description of methodology for TK, ICP4, ICP18.5 and ORFB-TK PCR RFLP is provided below from Kirkpatrick *et al.* (2006b) with some modifications.

For amplification of TK, PCR is performed in a 50- μ l reaction mixture containing 200 μ M each dATP, dCTP, dGTP, and dTTP, 1 mM MgCl₂, 250 μ M of each primer (TK forward: CTG-GGC-TAA-ATC-ATC-CAA-GAC-ATC-A; and TK reverse: GCT-CTC-TCG-AGT-AAG-AAT-GAG-TAC-A), 1.25 U of Taq DNA polymerase, 5 μ l of 10 $\times$ Taq DNA polymerase buffer, and 2 μ l of extracted DNA. The reaction mixture is incubated at 94°C for 3 minutes, then subjected to 35 cycles of 94°C for 15 seconds, 60°C for 45 seconds and 72°C for 60 seconds, ~~and finally incubated with the final cycle having an addition 3 minutes of incubation at 72°C for 3 minutes to ensure amplicons are double stranded for subsequent restriction digestion.~~

PCR amplification of ICP4, ICP18.5 and ORFB-TK, is performed in separate tubes using primers ICP4 forward: AAA-CCT-GTA-GAG-ACA-GTA-CCG-TGA-C and ICP4 reverse: ATT-ACT-ACG-TGA-CCT-ACA-TTG-AGC-C; ICP18.5 forward: TCG-CTT-GCA-AGG-TCT-TCT-GAT-GG and ICP18.5 reverse: AGA-AGA-TGT-TAA-TTC-ACA-CGG-ACA-C; and ORFB-TK forward: TCT-GCG-ATC-TTC-GCA-GTG-GTC-AG and ORFB-TK reverse: TGA-CGA-GGA-GAG-CGA-ACT-TTA-ATC-C. A 50- μ l reaction mixture contains 200 μ M each dATP, dCTP, dGTP, and dTTP, 2 mM MgSO₄, 250 μ M each primer, 1 U of Platinum Taq DNA Polymerase high fidelity, 5 μ l of 10 $\times$ Platinum Taq DNA polymerase buffer, and 2 μ l of extracted DNA. The reaction mixture is incubated at 94°C for 1 minute, then subjected to 35 cycles of 94°C for 1 minute, 68°C for 7 minutes, and finally incubated at 68°C for 10 minutes.

10- μ l volumes of TK, ICP4, ICP18.5 and ORFB-TK PCR products are digested separately with the restriction endonucleases MspI, HaeIII, HaeIII, and FokI respectively, at 37°C for 1 hour. After digestion, the resultant DNA fragments are separated in a 15% poly-acrylamide gel and restriction DNA fragments visualised by an appropriate nucleic acid stain and exposed to UV light for visualisation. Pattern differences are recorded for each enzyme and results can be developed into dendrograms.

2. Serological tests

The method of choice for detection of antibodies to ILTV in chicken serum is ELISA. Virus neutralisation (VN) can also be used (Adair *et al.*, 1985). AGID and indirect immunofluorescence tests are little used nowadays.

2.1. Enzyme-linked immunosorbent assay

A number of ELISA kits are available commercially, and the assay procedure may vary depending on the kit; the manufacturer's instructions should always be followed. Most commercial ELISAs use whole virus as detecting antigen (York *et al.*, 1983), but some use recombinant glycoproteins. For an in-house ELISA, the whole virus antigen can be obtained by sonication of heavily infected cell cultures at the time of maximum CPE, which is then centrifuged and the supernatant absorbed on to the wells of microtitre plates. A negative antigen is provided by uninfected cell culture material treated in the same way. The test consists essentially of the addition of 0.1 ml of 1/10 dilutions of test sera to duplicate wells coated with positive or negative antigen. After incubation at 37°C for 2 hours, the plates are washed four times and a rabbit anti-chicken IgG conjugated with peroxidase at a recommended dilution (1/1000 to 1/4000 depending on the manufacturer) is added. After incubation at 37°C for 1 hour, the plates are washed again four times. Finally, a chromagen consisting of 5-aminosalicylic acid is added to each well followed by hydrogen peroxide substrate to a final concentration of 0.0005%, and the absorbance of the fluid in each well is read at 450 nm on a spectrophotometer. The result for each serum is expressed as the difference between the mean absorbance produced with the positive and negative antigens. The positive/negative cut-off point is taken as the mean absorbance value for numerous negative sera plus 3 standard deviations. The test is very sensitive and may be useful for surveillance purposes. Antibody responses as measured by ELISA are detectable 7–10 days after infection and peak at about 2 weeks.

The response to ILT vaccines may be variable and testing is often done at 14 days post-vaccination or after.

2.2. Virus neutralisation

VN tests may be conducted on the dropped CAMs of embryonating chicken eggs that have been incubated for 9–11 days, where antibody specifically neutralises pock formation caused by ILTV. Alternatively, the tests can be performed in cell cultures, where antibody specifically neutralises the ILTV thus preventing CPE. Doubling dilutions of serum are added to equal volumes of a constant concentration of virus. This concentration may either be 100 median egg infectious doses (EID₅₀) for egg inoculations, or 100 median tissue culture infectious doses (TCID₅₀) for the inoculation of cultures. The mixtures are incubated at 37°C for 1 hour to allow any neutralisation to occur.

When the test is performed in eggs, the virus/serum mixtures are inoculated on to the dropped CAMs, using at least five eggs per dilution. Eggs are sealed and incubated at 37°C for 6–7 days. The end-point is recorded as the highest dilution of the serum where no pocks are present on the CAMs. When the tests are done in cell cultures, serum dilutions are prepared in 96-well microculture plates and virus is then added. After the period allowed for neutralisation, freshly prepared chicken embryo liver or kidney cells are added to each well. The plates are incubated at 37°C in an atmosphere of 5% CO₂ and examined daily for CPE; 50% end-points are read after approximately 4 days when the virus control titre indicates that 30–300 TCID₅₀ of virus have been used in the test. For the cell culture method of testing, virus neutralisation at 1/8 (initial dilution) or greater is considered positive.

C. REQUIREMENTS FOR VACCINES

1. Background

ILT is usually controlled with live vaccines. Vaccines may be used in response to disease outbreaks, or may be used routinely in endemic areas. Repeated doses may be required to afford good protection. Live attenuated ILT vaccines produced in cell cultures or embryonated hens' eggs are commonly used. Live recombinant (vectored) vaccines are also used. These vaccines use herpesvirus of turkeys or fowlpox virus as vectors to express ILTV proteins. There has also been some work with genetically engineered deletion-mutant vaccines and the results of these initial studies look promising (Coppo *et al.*, 2013). For attenuated ILT vaccines, the live virus seed is a suitably attenuated or naturally avirulent strain of ILTV. Live attenuated ILT vaccines may be administered by eyedrop, spray or in the drinking water. Recombinant vaccines may be delivered by wing-web puncture, subcutaneous injection or *in-ovo* inoculation. There are advantages and disadvantages associated with each of the different types of vaccine and the different methods of delivery (Coppo *et al.*, 2013; Garcia, 2017). For example, vectored vaccines may only be partially protective and live attenuated ILT vaccines may have residual virulence that can cause clinical disease, especially if administered by spray and a small droplet size is produced and inhaled. Live attenuated ILT vaccines may also revert to higher levels of virulence following bird-to-bird passage and persist in the field (Coppo *et al.*, 2013). For this reason, it may be difficult to discontinue vaccination once it has been started. Subclinical mixed infections of vaccine and field virus, in vaccinated birds, can cause severe disease in unvaccinated in-contacts. Natural recombination between attenuated vaccine strains of ILTV to produce virulent viruses has been reported and is also a risk for other live vaccines. The use of multiple different ILTV vaccines in the same populations should therefore be avoided (Coppo *et al.*, 2013).

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 (e.g. Code of Federal Regulations of the United States of America [USA], 2000; European Pharmacopoeia, 2010).

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics of the master seed

The virus seed for attenuated live ILT vaccines is a suitably attenuated or naturally avirulent strain of ILTV. Attenuation is achieved by serial passage of virus in embryonated eggs or tissue cultures. Recombinant vectored vaccines are created using recombinant DNA methodologies. The master seed virus (MSV) can be propagated in SPF chicken embryos or tissue cultures derived from such embryos. Initial tests are performed to demonstrate the safety and efficacy of the chosen master seed and the master seed must be tested for purity, virus identity and the presence of

extraneous pathogens. The MSV is stored in aliquots at -70°C . The viruses used in manufacture should be no more than five passages from the MSV.

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

Details on the origin of the virus seed, including passage history and any genetic manipulations should be recorded. The MSV is tested in chicken embryos or chickens for sterility and freedom from extraneous agents (see chapter 1.1.9). Virus content is determined by virus titration in chicken embryos or cell culture.

2.1.3. Validation as a vaccine strain

ILT vaccines cross-protect against different strains of ILTV. Infection of non-target species with vaccine strains of ILTV is not a major concern because of the narrow host range of ILTV.

2.2. Method of manufacture

2.2.1. Procedure

In large-scale vaccine production, the virus is propagated in SPF chicken embryos or tissue culture derived from such embryos, up to the fifth passage from the MSV. The acceptable passage level is supported experimentally by the passage level used to prepare the experimental product used in efficacy studies. The vaccine is made by inoculation of the production seed virus into 9- to 11-day-old chicken embryos or tissue culture prepared from chicken embryos derived from SPF flocks. Eggs are inoculated through a hole in the shell, on to the dropped CAM or into the allantoic sac. They are sealed and incubated at 37°C for 4–6 days. All eggs are candled before harvest and only those with living embryos are used. To harvest the virus, the eggs are chilled, then cleansed and opened aseptically. The CAMs or allantoic fluids are pooled in sterile, cooled containers. The CAMs should show the thick grey plaques typical of ILTV growth. Tissue culture-derived product would be prepared from virus-bearing cell culture fluids, which would also be subsequently pooled and tested.

2.2.2. Requirements for ingredients

Bovine serum used in vaccine production should be sourced from a country with negligible risk for transmissible spongiform encephalopathies [TSEs]. The origin of animal pancreatic trypsin used for cell culture should be considered. See chapter 1.1.8 with a special focus on products of biological origin originating from a country with negligible risk for TSEs.

2.2.3. In-process controls

The infected tissue or tissue culture homogenate may be tested for purity, potency, virus content and safety.

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

The presence of ILTV may be confirmed by mixing the vaccine with ILTV antiserum and demonstrating that it is no longer able to infect embryonated hens' eggs from an SPF flock, or susceptible cell lines into which it is inoculated.

iii) Safety

Each batch of vaccine is delivered by eyedrop (10 doses per bird) or by intratracheal injection to SPF chickens, or other target species. The birds are observed for 14–21 days for adverse effects attributable to the vaccine.

iv) Batch potency

Once the *in-vivo* efficacy of the vaccine has been established, the batch potency may be determined by measuring the virus content. This is done by virus titration using serial dilutions of vaccine inoculated onto the dropped CAM of embryonated hens' eggs, or inoculation of suitable cell cultures. The virus content (tested at any time within the expiration

period) should be at or above the minimum titre stated on the label. The release and expiration titres are based on the minimum protective dose of the vaccine. Although efficacy testing of each vaccine batch may not be required, efficacy testing of a representative batch (using a vaccinating dose containing not more than the minimum virus titre stated on the label) may be needed.

2.3. Requirements for regulatory approval

2.3.1. Manufacturing process

For approval 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

In the USA, 25 susceptible chickens, 3- to 4-weeks old, are injected intratracheally and observed for 14 days. Deaths are counted as failures. Four or fewer failures are allowed for satisfactory serials. In the European Union (EU), initial safety tests use the vaccine virus at the least attenuated passage level between the MSV and a batch of the vaccine. Twenty SPF chickens are inoculated with 10 times the vaccine dose and observed for 21 days. Each route and method of administration that is recommended for vaccination should be tested in chickens at the youngest age recommended for vaccination. No notable clinical signs or deaths attributable to ILT are allowed. Batch tests for safety in the EU require eye-drop inoculation of 10 SPF chickens, at the youngest age recommended for vaccination, with 10 times the vaccine dose. Chickens are observed for 21 days. No notable clinical signs or deaths attributable to ILT are allowed.

i) Reversion to virulence

The EU requires five *in-vivo* sequential passages of vaccine virus. Each passage is performed in groups of five SPF chickens. Sequential passage may be achieved by natural spreading, or by collecting and pooling infectious material from the respiratory tract of one group of birds and using this material to infect the subsequent group of birds via eye-drop inoculation. Passaged virus recovered after five sequential passages (or earlier if passage cannot be maintained) is compared with the unpassaged virus using an index of respiratory virulence test. The maximally passaged virus must show no increase in virulence. The index of respiratory virulence tests involves intratracheal inoculation of 0.2 ml of vaccine at three different doses, starting with stock that has titre of 10^5 EID₅₀ or 10^5 CCID₅₀ per 0.2 ml (or maximal obtainable titre) and then two serial tenfold dilutions of that stock. At least 20 SPF chickens are inoculated per dose. Chickens are observed for 10 days and deaths are recorded. The index of respiratory virulence is the total number of deaths observed in the three groups divided by the total number of chickens

ii) Precautions (hazards)

Care should be taken over diluting and administering the vaccine, and over the proper disposal of unused vaccine.

2.3.3. Efficacy requirements

A test must be carried out to establish the efficacy of the vaccine in groups of birds of the minimum age for which the product is destined and also for each avian species. Each route and method of administration recommended for vaccination must be tested. Twenty (USA) or 30 (EU) chickens are vaccinated with the most attenuated passage level of vaccine virus. Ten additional chickens are held as controls. After 10–14 days (USA) or 21 days (EU) vaccinated chickens and controls are challenged with virulent ILTV intratracheally or in the orbital sinus. At least 80% (USA) or 90% (EU) of unvaccinated controls must die or show severe clinical signs of ILT, or show notable macroscopic lesions. To be satisfactory, only 5% or 10% of the vaccinated birds should die or show severe clinical signs of ILT, or show notable macroscopic lesions.

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

The advent of recombinant (vectored) ILT vaccines has brought the potential to differentiate serologically between infected and vaccinated birds and the potential to use DIVA control strategies. Many of the gene-deleted ILT vaccines that are currently under development offer the same potential. A number of studies have reported the use of serological screening tools to detect antibodies against specific ILTV glycoproteins (Coppo *et al.*, 2013).

2.3.5. Duration of immunity

The results of vaccination will depend on many factors, including dose schedule and route of administration. Some degree of protection should be given, over a period of several months.

2.3.6. Stability

Stability is tested by taking samples of correctly stored vaccine at intervals and measuring virus content. Tests should be carried out on at least six batches of the vaccine or until a statistically valid number of serials have been evaluated and be continued for 3 months after the claimed shelf-life.

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NB: At the time of publication (2025) there were no WOA Reference Laboratories for avian infectious laryngotracheitis (please consult the WOA Web site:

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

NB: FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2021.

Appendix 1: Avian infectious laryngotracheitis
Intended purpose of test: population freedom from infection

<u>Test with score and species: chicken</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>PCR: ☒☐☐</u>	<u>Tracheal swabs or scrapings</u> <u>Various gene targets</u>	<u>DSp 0.18 DSe 1.0 limit of quantitation</u> <u>100 template copies</u>	<u>346 naturally and experimentally infected birds, and 680 negative birds</u>	<u>See reference</u>	<u>Rapid; sensitive; accurate</u>	<u>Expensive; a large number of individuals will need to be tested for this intended purpose as birds are often asymptomatic</u>	<u>Callison <i>et al.</i> (2007)</u>
<u>VN: ☒☐☐</u>	<u>Serum, antibody</u> <u>Whole virus</u>	<u>Statistical analysis not conducted</u>	<u>5 vaccinated, 20 naturally infected</u>	<u>No validation report found</u>	<u>Accurate</u>	<u>Time consuming; restricted by the availability of fertile eggs and cells, expensive</u>	<u>Adair <i>et al.</i> (1985)</u>
<u>ELISA – antibody detection: ☒☒☐</u>	<u>Serum antibody</u> <u>Whole virus</u>	<u>Statistical analysis not conducted</u>	<u>174 negative, 8 inoculated chickens</u>	<u>See reference</u>	<u>Rapid; sensitive</u>	<u>Can have nonspecific positive reactions; antibodies take 2-3 weeks after infection to reach a detectable level</u>	<u>York <i>et al.</i> (1983)</u>

Appendix 2: Avian infectious laryngotracheitis
Intended purpose of test: individual animal freedom from infection prior to movement

<u>Test with score and species: chicken</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>ELISA – antigen detection: ☒☒☐</u>	<u>Tracheal scrapings/swabs</u> <u>monoclonal antibody</u>	<u>Statistical analysis not conducted</u>	<u>13 positive samples</u> <u>25 negative samples</u>	<u>See reference</u>	<u>Relatively rapid</u>	<u>Not widely available</u>	<u>York & Fahey (1988)</u>
<u>PCR: ☒☒☒</u>	<u>Tracheal swabs</u> <u>Various gene targets</u>	<u>D_{Sp} 0.18 D_{Se} 1.0 limit of quantitation</u> <u>100 template copies</u>	<u>346 naturally and experimentally infected birds, and 680 negative birds</u>	<u>See reference</u>	<u>Rapid; sensitive; accurate</u>	<u>Expensive</u>	<u>Callison <i>et al.</i> (2007)</u>
<u>ELISA – antibody detection: ☒☐☐</u>	<u>Serum antibody</u> <u>Whole virus</u>	<u>Statistical analysis not conducted</u>	<u>174 negative, 8 inoculated chickens</u>	<u>See reference</u>	<u>Rapid; sensitive</u>	<u>Can have nonspecific positive reactions; antibodies take 2–3 weeks after infection to reach a detectable level</u>	<u>York <i>et al.</i> (1983)</u>

Appendix 3: Avian infectious laryngotracheitis
Intended purpose of test: contribute to eradication policies

<u>Test with score and species: chicken</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>PCR: ☒☒☐</u>	<u>Tracheal swabs or scrapings</u> <u>Various gene targets</u>	<u>DSp 0.18 DSe 1.0 limit of quantitation</u> <u>100 template copies</u>	<u>346 positive naturally and experimentally infected birds, and 680 negative birds</u>	<u>See reference</u>	<u>Rapid; sensitive; accurate</u>	<u>Expensive; a large number of individuals will need to be tested for this intended purpose as birds are often asymptomatic</u>	<u>Callison <i>et al.</i> (2007)</u>
<u>VN: ☒☐☐</u>	<u>Serum, antibody</u> <u>Whole virus</u>	<u>Statistical analysis not conducted</u>	<u>5 vaccinated, 20 naturally infected</u>	<u>No validation report found</u>	<u>Accurate</u>	<u>Time consuming; restricted by the availability of fertile eggs and cells, expensive</u>	<u>Adair <i>et al.</i> (1985)</u>
<u>ELISA – antibody detection: ☒☒☒</u>	<u>Serum antibody</u> <u>Whole virus</u>	<u>Statistical analysis not conducted</u>	<u>174 negative, 8 inoculated chickens</u>	<u>See reference</u>	<u>Rapid; sensitive</u>	<u>Can have nonspecific positive reactions; antibodies take 2–3 weeks after infection to reach a detectable level</u>	<u>York <i>et al.</i> (1983)</u>

Appendix 4: Avian infectious laryngotracheitis
Intended purpose of test: confirmation of clinical cases

<u>Test with score and species: chicken</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Virus isolation:</u> ☒ ☒ ☐	<u>Tracheal scrapings or tracheal swabs</u> <u>CAMS of embryonated chicken eggs</u>	<u>Statistical analysis not conducted</u>	<u>11 samples from clinically affected farms.</u>	<u>See reference</u>	<u>Isolate can be used for further typing</u>	<u>Slow; requires available embryonated eggs or cells</u>	<u>Hughes & Jones (1998)</u>
<u>Immuno-fluorescence for antigen:</u> ☒ ☒ ☐	<u>Tracheal scrapings</u> <u>ILTV Antiserum</u>	<u>Statistical analysis not conducted</u>	<u>33 flocks clinically affected. 22 flocks with no clinical disease</u>	<u>See reference</u>	<u>Rapid</u>	<u>Lacks sensitivity; needs fluorescent microscope</u>	<u>Wilks & Kogan (1979)</u>
<u>ELISA – antigen detection:</u> ☒ ☒ ☒	<u>Tracheal scrapings/swabs</u> <u>Monoclonal antibody</u>	<u>Statistical analysis not conducted</u>	<u>13 positive samples 25 negative samples</u>	<u>See reference</u>	<u>Relatively rapid</u>	<u>Not widely available</u>	<u>York & Fahey (1988)</u>
<u>PCR:</u> ☒ ☒ ☒	<u>Tracheal swabs or scrapings</u> <u>Various gene targets</u>	<u>DSp 0.18 DSe 1.0</u> <u>Limit of quantitation</u> <u>100 template copies</u>	<u>346 naturally and experimentally infected birds, and 680 negative birds</u>	<u>See reference</u>	<u>Rapid; sensitive, accurate</u>	<u>Expensive</u>	<u>Callison <i>et al.</i> (2007)</u>
<u>Histopathology:</u> ☒ ☒ ☐	<u>Larynx or trachea</u> <u>H&E staining of the paraffin embedded tissues</u>	<u>Statistical analysis not conducted</u>	<u>Historical validation</u>	<u>No validation report found</u>	<u>Useful for confirmation of infection</u>	<u>Specific microscopic lesions (intranuclear inclusion bodies) and syncytial cell formations may be present only for a short period of time</u>	<u>Garcia & Spatz (2020)</u>
<u>ELISA – antibody detection:</u> ☒ ☐ ☐	<u>Serum antibody</u> <u>Whole virus</u>	<u>Statistical analysis not conducted</u>	<u>174 negative, 8 inoculated chickens</u>	<u>See reference</u>	<u>Rapid; sensitive</u>	<u>Can have nonspecific positive reactions; not useful at early stages of the infection as antibodies take 2–3 weeks after infection to reach a detectable level</u>	<u>York <i>et al.</i> (1983)</u>

Appendix 5: Avian infectious laryngotracheitis
Intended purpose of test: prevalence of infection – surveillance

<u>Test with score and species: chicken</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>PCR: ☒☒☒</u>	<u>Tracheal swabs or scrapings</u> <u>Various gene targets</u>	<u>DSp 0.18 DSe 1.0 Limit of quantitation</u> <u>100 template copies</u>	<u>346 naturally and experimentally infected birds, and 680 negative birds</u>	<u>See reference</u>	<u>Rapid; sensitive; accurate</u>	<u>Expensive; a large number of individuals will need to be tested for this intended purpose as birds are often asymptomatic</u>	<u>Callison <i>et al.</i> (2007)</u>
<u>ELISA – antibody detection: ☒☒☒</u>	<u>Serum antibody</u> <u>Whole virus</u>	<u>Statistical analysis not conducted</u>	<u>174 negative- and 8 inoculated chickens</u>	<u>See reference</u>	<u>Rapid; sensitive</u>	<u>Can have nonspecific positive reactions; antibodies take 2–3 weeks after infection to reach a detectable level</u>	<u>York <i>et al.</i> (1983)</u>

Appendix 6: Avian infectious laryngotracheitis
Intended purpose of test: immune status in individual animals or populations post-vaccination

<u>Test with score and species: chicken</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>VN: ☒☒☐</u>	<u>Serum, antibody</u> <u>Whole virus</u>	<u>Statistical analysis not conducted</u>	<u>5 vaccinated,</u> <u>20 naturally infected</u>	<u>No validation report found</u>	<u>Accurate; is thought to be highly correlated with protective immunity</u>	<u>Time consuming; restricted by the availability of fertile eggs and cells; expensive</u>	<u>Adair <i>et al.</i> (1985)</u>
<u>ELISA – antibody detection: ☒☒☒</u>	<u>Serum antibody</u> <u>Whole virus</u>	<u>Statistical analysis not conducted</u>	<u>174 negative,</u> <u>8 inoculated chickens</u>	<u>See reference</u>	<u>Rapid; sensitive</u>	<u>Can have nonspecific positive reactions; antibodies take 2–3 weeks after infection to reach a detectable level; serum antibodies may not necessarily correlate with protective immunity</u>	<u>York <i>et al.</i> (1983)</u>

MEETING OF THE BIOLOGICAL STANDARDS COMMISSION

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CHAPTER 3.3.5.

AVIAN MYCOPLASMOSIS
(*MYCOPLASMA GALLISEPTICUM*, *M. SYNOVIAE*)

SUMMARY

Description of the disease: Avian mycoplasmosis is caused by several pathogenic mycoplasmas among which *Mycoplasma gallisepticum* (MG) and *M. synoviae* (MS) are considered the most ~~important-significant~~. MG causes chronic respiratory disease of domestic poultry, especially when flocks are stressed and/or other respiratory pathogens are present. The disease is characterised by coryza, conjunctivitis, sneezing, and sinusitis, particularly in turkeys and game birds. It can result in significant production losses and downgrading of meat-type birds, and loss of egg production. MS may cause respiratory disease, synovitis, eggshell alteration, loss of egg production and carcass downgrading ~~or it may result in a silent infection~~. MG and MS strains vary in infectivity and virulence, and infections may sometimes be inapparent.

Detection of the agent: MG and MS can be identified by immunological methods after bacterial isolation in mycoplasma media or by detection of their DNA in field samples or cultures.

Samples suitable for bacterial isolation can be swabs of organs or tissues, exudates, diluted tissue homogenates, aspirates from the infraorbital sinuses or joints ~~cavities~~, material from egg yolk or embryos. Clinical signs and lesions will influence the sample selection. Broth and agar combined with basic biochemical tests are used for isolation and first recognition of the *Mycoplasma*, but identification of the genus and species is made through immunological tests (e.g. fluorescent antibody or immunoperoxidase tests) and/or biomolecular tests.

DNA detection methods based on the polymerase chain reaction are ~~commonly used in several specialised laboratories available~~.

Serological tests: Several serological tests are used to detect MG or MS antibodies, but due to variations in test specificity and sensitivity, they are recommended only for flock screening rather than for testing individuals.

The most commonly used are the rapid serum agglutination (RSA) test, the enzyme-linked immunosorbent assay (ELISA) and the haemagglutination inhibition (HI) test. In the RSA test, sera are mixed with commercially produced stained antigen, ~~and~~ Sera that react within 2 minutes are heated at 56°C for 30 minutes and retested. Sera that still react, especially when diluted, are considered positive and are ~~tested by confirmed using~~ either ELISA or HI ~~for confirmation~~. Several commercial MG and MS antibody ELISA kits are available.

Requirements for vaccines: Although the preferred method of control is ~~maintenance of~~ maintaining MG- and MS-free flocks, both live and inactivated vaccines are used in chickens. Vaccination should be considered only in specific cases ~~on the basis of~~ based on the epidemiological situation in the area or in farms where infection is ~~inevitable-unavoidable~~. ~~The normal use is~~ It is primarily used to prevent egg-production losses in commercial layers, ~~although vaccines may also be used to~~ though it may also help reduce egg transmission in breeding stock or ~~to aid support~~ MG eradication on multi-

age sites. ~~It is important~~ To be effective, vaccination must occur ~~vaccinate~~ before field challenge occurs ~~exposure~~.

~~Available~~ Live vaccines for MG for chickens are produced ~~derived~~ from the F strain and strains as well as the ts-11 and 6/85 strains. The latter two ~~which are~~ apathogenic and offer improved safety profiles ~~strains with improved safety characteristics. More recently, another live vaccine derived from the K strain was released.~~ Administration of the F strain by the intranasal or eyedrop route is preferred, but aerosol or drinking water administration may be used. For ts-11, the eyedrop method is recommended ~~for ts-11, and while a fine spray is preferred for 6/85.~~ Pullets are generally vaccinated between 9 and 16 weeks of age. ~~One~~ A single dose is sufficient and vaccinated birds remain permanent carriers. Long-term use of the F strain on multi-age sites can result in displacement of field strains. The ts-11 strain has been successfully used to eradicate the F strain in multi-age commercial layers. In the past few years, two live MS vaccines produced from the MS-H strain and MS1 have been licensed in several countries, although the latter is not globally available on the market. Eyedrop administration is advised for the MS-H strain whereas fine aerosol is recommended for MS1. ~~The~~ Birds should be vaccinated by 5 weeks of age. New MG vaccine candidates are under study, some of them are attenuated strains and others are based on vectored-virus technology.

Inactivated vaccines consisting of concentrated suspension of MG or MS organisms in an oil emulsion are licensed in several countries. They should be administered parenterally to pullets at 12–16 weeks of age. Two doses are desirable. MG bacterins are effective in preventing egg-production losses and respiratory disease, but do not prevent infection with wild-type MG. MS inactivated vaccines are not ~~in~~ commonly used.

A. INTRODUCTION

1. Description and impact of the disease

Mycoplasma gallisepticum (MG) and *M. synoviae* (MS), members of ~~belong to~~ the class Mollicutes, order Mycoplasmatales, family Mycoplasmataceae, are distributed worldwide and are considered the most important avian pathogenic mycoplasmas. It should be noted, however, that *M. meleagridis* and *M. iowae* can also cause disease in poultry, but MG and MS are considered to be the most important avian pathogenic mycoplasmas, and both occur world-wide.

MG infection is particularly important in chickens and turkeys, in which the pathogen causes ~~as a cause of~~ respiratory disease and decreased meat and egg production (Raviv & Ley, 2013). ~~It~~ This mycoplasma can also cause upper respiratory disease in game birds. Some strains of MG have been recognised in North America identified in house finches in North America as a cause of conjunctivitis, leading to significant ~~with important~~ impacts in ~~on~~ wild bird populations, including difficulty finding ~~such as inability to find food, starvation, and or death,~~ However, these strains ~~but they~~ do not appear to be pathogenic for ~~to~~ poultry (Ferguson et al., 2020).

In poultry the infection is spread vertically through infected eggs and horizontally by close contact; the MG nucleic acid has been identified in environmental samples. ~~Recently~~ It was shown that MG and MS may survive up to 9 days in synthetic fibres though ~~less in their survival is shorter on~~ human hair (Abolnik & Gouws, 2014), indicating a tendency for ~~showing a predisposition of these mycoplasmas for attachment to~~ adhere to surfaces. Other modes of transmission remain ~~methods of spread are~~ less well documented.

The clinical signs of MG infection in ~~infected~~ poultry can vary from subclinical to obvious respiratory signs including coryza, conjunctivitis, coughing and sneezing. Nasal exudate, rales and breathing through the partially open beak may occur. Unilateral or bilateral sinusitis may also ~~be a feature~~ occur, particularly in turkeys and game birds, with swelling of and the infraorbital sinuses ~~sometimes becoming severe enough to cause eyelid closure may become so swollen that the eyelids are closed.~~ Conjunctivitis, with ~~accompanied by~~ frothy ocular exudate is also seen in turkeys, game birds and, ~~sometimes occasionally,~~ in chickens. In turkeys, there is often soiling of the wing feathers ~~soiling is often seen due to repeated as the result of attempts to remove~~ wipe exudate from the eyes. Infected finches may also exhibit conjunctivitis along with ~~reveal~~ ocular and nasal discharge, as well as ~~and~~ swollen eyelids in addition to the conjunctivitis.

MG ~~may be associated with acute respiratory disease in chickens and turkeys, especially in young birds, with the turkey being more susceptible. The severity of the disease is greatly influenced by the degree of secondary infection with viruses such as Newcastle disease and infectious bronchitis, and/or bacteria such as Escherichia coli.~~ During MG infection, environmental factors such as the presence of dust and ammonia, cold weather, and overcrowded housing play an important role in the increased number of lesions. Additionally, other upper respiratory pathogens (such as Newcastle disease virus [NDV], infectious bronchitis virus [IBV], influenza A virus, infectious laryngotracheitis virus, Haemophilus paragallinarum, and Escherichia coli), as well as live virus vaccinations also

contribute to the exacerbation of MG infection clinical signs. In turkeys, there is synergism with avian metapneumovirus infection. A more chronic form of the disease may occur resulting in reduced egg production in breeders and layers.

Lesions of the respiratory tract initially present as excessive mucous exudate, progressing to followed by catarrhal and caseous exudate, which may ~~form-accumulate as~~ amorphous masses in the air sacs. In turkeys and game birds the swollen infraorbital sinuses typically contain mucoid to caseous exudate.

MS may be associated in chickens with infectious synovitis; the birds may exhibit pale combs, lameness and retarded growth. Swellings may occur around joints. Greenish droppings containing large amounts of urates are commonly seen. Joints may contain a viscous, creamy to grey exudate ~~in the joint, which can also extend and along the tendon sheaths,-~~. Additionally, along with hepatosplenomegaly and mottled, swollen kidneys may be observed (Ferguson-Noel & Noormohammadi, 2013). Respiratory signs and lesions are similar to those observed with MG, except that they are generally milder, and, as with MG, there is a synergistic effect with other respiratory agents. MS strains exhibit significant variability with respect to their virulence and tissue tropism ~~(Catania et al., 2016a; Landman, 2014; Landman & Feberwee, 2004).~~ Recently Between 2009 and 2010, a new clinical form resulting in high shell breakage and low egg production in chicken layers was reported initially in Europe ~~(Catania et al., 2010; Feberwee et al., 2009a; 2009b)~~ and then reported worldwide (Ferguson et al., 2020). The lesions appear to be confined to the apex of the shell and consist of rough dark areas of 2 cm in diameter with clear edges; in addition, a decrease in egg production is reported ~~(Catania et al., 2010; 2016a).~~

Recently, it has been proved that both MG and MS field strains may be capable of producing biofilm in vitro (Catania et al., 2024). However, further studies focused on this biological feature are needed to determine the influence of biofilm formation in the epidemiology of pathogenic avian mycoplasmas.

2. Zoonotic potential and biosafety and biosecurity requirements

There have been no reports of MG or MS infection in humans. Laboratory manipulations should be performed with appropriate biosafety and containment procedures as determined by biological risk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

3. Differential diagnosis

MG or MS disease in chickens may ~~superficially-closely~~ resemble respiratory disease caused by other pathogens such as mild strains of Newcastle disease virus (Chapter 3.3.14) and avian infectious bronchitis virus (Chapter 3.3.2). These viruses may be also present in mixed infections with MG or MS. Infections with *Avibacterium paragallinarum* and *Pasteurella multocida* should also be ruled out. In broilers, the co-infection with avian metapneumovirus and *E. coli* ~~could show may present with~~ similar ~~features-signs of disease.~~ MG infections in turkeys may be confused with avian metapneumovirus infections and the presence of sinusitis may also suggest infection with *Bordetella avium*, *Pasteurella multocida*, *Chlamydia* (Chapter 3.3.1) or MS. Infectious synovitis caused by MS should be differentiated from infections with *Staphylococcus aureus*, *Riemerella anatipestifer*, *Ornithobacterium rhinotracheale* and *Enterococcus*, as well as ~~joint infections and, in chicken, from~~ infectious tenosynovitis caused by avian orthoreoviruses in chickens.

B. DIAGNOSTIC TECHNIQUES

The presence of MG or MS can be confirmed by isolating the organism in a cell-free medium or by detecting its DNA directly in infected tissues or swabs ~~samples~~. Serological tests are also widely used for diagnosis. When results are inconclusive, equivocal the birds are usually resampled for further testing.

Table 1. Test methods available for diagnosis of avian mycoplasmosis (*Mycoplasma gallisepticum*, *M. synoviae*) 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)						
Conventional PCR	☑☑☐ ^(h)	☑☑☐ ^(h)	☑☑☐ ⁽ⁱ⁾	☑☑☑	☑☑☐ ^(j)	☐☐☐

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)
Real-time PCR	✓✓✓✓ ✓✓✓✓ ^(h)	✓✓✓✓ ✓✓✓✓ ^(h)	✓✓✓✓ ^(a)	✓✓✓✓	✓✓✓✓ ^(a)	□□□
Isolation on culture media	✓□□ ^(h)	□□□	✓□□ ^(a)	✓✓✓✓-✓□□ ^(a)	□□□	□□□
PCR-DGGE ⁽ⁱ⁾	✓□□	□□□	✓□□ ^(a)	✓□□	□□□	□□□
Detection of immune response						
HI	✓✓✓ ⁽ⁱ⁾	□□□	✓□□	✓✓✓ ⁽ⁱ⁾	✓✓✓ ^(a)	✓✓✓✓-✓□□ ^(a)
RSA	✓□□ ^(k)	□□□	✓□□	✓□□ ^(l)	✓□□ ^(a)	✓□□
ELISA	✓✓✓ ⁽ⁱ⁾	□□□	✓✓✓	✓✓✓ ^(l)	✓✓✓✓ ✓✓✓ ^(a)	✓✓✓ ^(m)

Key: ✓✓✓ = recommended for this purpose; ✓✓□ = recommended but has limitations;

✓□□ = suitable in very limited circumstances; □□□ = not appropriate for this purpose.

PCR: polymerase chain reaction; DGGE = denaturing gradient gel electrophoresis; HI = hemagglutination inhibition;

RSA = rapid serum agglutination; ELISA = enzyme-linked immunosorbent assay.

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

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

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

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

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

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

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

^(h)Not suitable for day old birds; ⁽ⁱ⁾applied in culture medium, isolated colonies;

^(j)Suitable for ensuring lack of infections dating back more than 2–3 weeks;

^(k)Suitable for ensuring lack of infections dating back more than 5–8 days;

^(l)Suitable provided paired samples collected from non-vaccinated animals a few weeks apart can be analysed;

^(m)Suitable only for the group vaccinated with killed vaccine, F strain, K strain and by temperature sensitive vaccines

⁽ⁿ⁾Suitable only for non-vaccinated groups.

1. Detection of the agent

1.1. *In-vitro* culture

Samples are taken from live birds or, fresh carcasses ~~or the carcasses~~ of birds that have been frozen quickly after death. In live animals, the tracheal swab is swabs are generally considered to be the best most reliable sample in live animals type for detecting most mycoplasma species. In addition, for isolation purposes, it is possible to collect swabs from the choanal cleft. When dead birds are available, mycoplasma isolation can be performed with swabs from different tissues or organs such as the upper and/or mid-trachea, lungs, air sacs, oviduct and joints.

Swabs of the yolk sac should be collected during the last third of the ~~egg~~-incubation period ~~where~~-when decreased hatchability of embryonated eggs has occurred (i.e. after the 15th and the 20th day of incubation in chickens and in turkeys, respectively).

Samples can be taken from the inner surface of the vitelline membrane, and from the oropharynx and air sacs of the embryo.

All samples should be ~~tested~~-processed in the laboratory as soon as possible after collection. If transportation is necessary, collected swabs should be ~~vigorously agitated~~-swirled in 1–2 ml of mycoplasma broth and then discarded; tissues or organs should be kept frozen until sampling. An ice pack or some other means of chilling should be included as MG and MS die rapidly at room temperature. Serial dilutions of samples should be made in mycoplasma broth because the presence of specific antibodies, antibiotics or inhibitory substances in tissues may inhibit mycoplasma growth.

Several suitable culture media have been formulated to support the growth of avian mycoplasmas; in addition, ~~several many~~ commercial media are also available. Mycoplasma media generally contain a digested proteins and a meat-infusion base supplemented with serum or a serum fraction, yeast factors, glucose and bacterial inhibitors. It is important that each new batch of medium be tested with recently isolated MG cultures of low *in-vitro* passage because some components, especially the yeast extract and the blood serum may vary in their ability to support growth.

The medium developed by Frey *et al.* is widely used ~~in the United States of America (USA) and other countries~~ for isolation of MG and MS (Frey *et al.*, 1968). Nicotinamide adenine dinucleotide (NAD) is a growth requirement for the primary isolation of MS, but it may be omitted in the medium for the cultivation of MG.

The following broth and agar media are also satisfactory:

- i) Part A: Pleuropneumonia-like organism (PPLO) broth base without crystal violet (44.7-21 g); distilled or deionised water (700 ml).
- ii) Part B: Pig serum (heated at 56°C for 1 hour) (150 ml); 25% (w/v) fresh yeast extract (100 ml); 10% (w/v) glucose solution (10 ml); 5% (w/v) thallium acetate (10 ml); 200,000 International Units (IU)/ml penicillin G (5 ml); and 0.1% (w/v) phenol red solution (20 ml). Thallium acetate can be toxic to humans and the precautions for its use should be followed. The pH is adjusted to 7.8. Pig serum may be replaced by horse serum, but it is important to ascertain that it some laboratories use a mix of the two types of sera; however it is advised to verify that the selected serum batch supports the growth of fastidious MG strains. For primary isolation of MS in this medium, a mix of 10% (v/v) gNAD solution (4-10 ml) and 10% (v/v) cysteine solution (4-10 ml) is also added.

Part A is autoclaved at 121°C, at 1 atmospheric pressure for 15 minutes and, after cooling, is added to Part B, which has previously been sterilised by filtration.

For the corresponding solid medium, 10 g of purified agar, known to support the growth of mycoplasma, is added to part A ~~above~~. The mixture is autoclaved as ~~before described above~~ and then it is kept in a water bath at 56°C. The constituents of part B, omitting the phenol red, are mixed separately and then incubated at 56°C. Parts A and B are mixed carefully to avoid the production of air bubbles, and are dispensed into 50 mm dishes using 7–9 ml/dish. Excess surface moisture can be removed by a short incubation at 37°C. Plates are stored in an airtight container at approximately 4°C for up to 4 weeks.

Fresh yeast extract is available commercially, although it is preferable to prepare it 'in-house' by taking active dry baker's yeast (250 g) and suspending it in distilled water (1 litre). ~~This The suspension~~ is heated to boiling point, cooled and then centrifuged for 20 minutes at 3000 g. The supernatant fluid is decanted and adjusted to pH 8.0 with 0.1 M NaOH. This is clarified by centrifugation or by filtration, and then sterilised by filtration. The extract is stored at –20°C. Reagent grade glucose (10 g) is dissolved in distilled or deionised water (100 ml) and adjusted to pH 7.8–8.0 with 0.1 M NaOH. It is sterilised by filtration and stored at 4°C. Reagent grade thallium acetate is dissolved (5 g) in distilled or deionised water (100 ml), filter-sterilised and stored at –20°C. Penicillin solution (10⁶ IU benzyl penicillin in 5 ml distilled water) is stored at 4°C, and has a shelf life of 1 week. For isolation from heavily contaminated samples, penicillin concentration can be increased to 2000 units/ml or ampicillin, 0.5–1.0 mg/ml, may be used instead. Phenol red (0.1 g) is ground in 0.1 M NaOH (2.8 ml), and then made up to 100 ml in sterile distilled water and autoclaved at 115°C at 1 atmosphere for 30 minutes. It is stored at 4°C. (Note: thallium acetate is highly toxic and care should be taken, especially when preparing the stock solution.)

There are other media available for culturing mycoplasma. Some of these can be prepared in-house, while others are commercially available from private companies.

Specimens are inoculated onto both mycoplasma agar and into broth. The use of solid medium may help detection of aid in detecting slow-growing mycoplasmas, which ~~can may otherwise~~ be overgrown by ~~saprophytes saprophytic organisms~~ in broth cultures. To improve the chances of successful isolation, it may be necessary to make serial dilutions up to 10⁻³ may be necessary for successful isolation. Inoculated agar plates are sealed with parafilm strips and incubated at 37°C in sealed containers. Increased humidity and CO₂ ~~tension in the atmosphere levels~~ have been reported to enhance mycoplasma growth. These conditions may be obtained achieved by: ~~the inclusion of~~ including damp paper or cotton wool, flushing the container with 5–10% CO₂ in nitrogen, placing a lighted candle in the container, or ~~by using~~ a CO₂ incubator or suitable gas-generating system.

The caps of ~~liquid medium containers broth culture tubes~~ should be tightly sealed before incubation at 37°C to avoid spurious changes in pH. For the first few days, the plates are examined daily for ~~colonies~~ colony development with a stereoscopic microscope; after that, ~~they are examined~~ less frequently

observation is sufficient. Cultures derived from field material should be kept for at least 20 days before discarding-being discarded.

Broth cultures should be examined daily for changes in colour and/or in turbidity. Most mycoplasmas, including MG and MS, metabolise sugars to produce producing acid, resulting in causing a change in the pH of the medium from red/orange to yellow. Other mycoplasmas hydrolyse arginine, creating alkaline conditions causing a change in pH and consequently of and a shift in broth colour from red/orange to strong red or fuchsia. Any observable growth in broth is immediately subcultured onto solid medium immediately. If there is no colour change after 7–14 days, the broth should be subcultured onto solid medium. This should be done is important because the presence of an arginine-hydrolysing (alkali-producing) mycoplasma species may mask the acid-induced colour change produced by MG or MS, or because there might be mycoplasma strains with less active metabolism-weak metabolic activity.

Mycoplasma colonies on solid medium can are usually easily be recognised, although they may not have the typical 'fried egg' appearance. Bacterial colonies other than mycoplasma may appear on the first passage, but they are often more pigmented and fail to passage on mycoplasma media.

Biochemical reactions (e.g. fermentation of glucose and failure to hydrolyse arginine) can assist in identification, but they are not specific for MG or MS and necessitate purification of the culture by cloning.

Immunological and DNA detection methods can be used to identify mycoplasma isolates. They include the growth inhibition (GI), and metabolism inhibition (MI), indirect fluorescent antibody (IFA), and immunoperoxidase (IP) tests. The latter two can be considered easy to perform-simple, sensitive and specific. Pure cultures (produced by generated from one single colony) are required for the GI and MI tests, but not for the IFA or IP test. IFA and IP can detect multiple the presence of more than one species of-mycoplasma species, as only the colonies that are reactive with the specific for the antiserum will produce a positive signal-react. However, *M. imitans* = a mycoplasma-species that is serologically and biochemically similar to MG = has been isolated from ducks, geese and sometimes from other non-domestic bird species in some-certain countries. It may be distinguished from MG by-using of specific biomolecular methods. Alternatively, isolates may be differentiated by IFA colonies of the isolate can be examined by immunofluorescence using serial dilutions of antisera to both MG and *M. imitans* in parallel; the homologous antiserum should have a considerably higher titre.

DNA detection methods for identifying MG or MS directly in tissues or for identifying laboratory isolates are discussed below and are usually based on the polymerase chain reaction (PCR).

In certain circumstances where results of the above methods are not conclusive, inoculation of chick embryos or bioassays in live chicks may be appropriate. However, these techniques are time-consuming and costly, and have largely been replaced by PCR technology, although they remain a useful research tool. The specimens required for inoculation of chicken embryos are the same as those used for the inoculation of artificial media. They are prepared in broth from which thallium acetate is omitted, incubated for 30–60 minutes at 37°C, and then a 0.05–0.1 ml aliquot is inoculated into the yolk sac of several 6- to 8-day-old chicken embryos derived from mycoplasma-free flocks. The eggs are candled daily and embryos that die within 24 hours of inoculation are discarded. Any further dead embryos are kept refrigerated until cultured and those surviving after 5 days are placed at 4°C for 4 hours to kill them and to reduce haemorrhages on opening. The yolk is subcultured into broth and on to agar. Yolk lipid tends to obscure colonies, so it is essential to streak the yolk thinly or, preferably, to dilute it first in mycoplasma broth.

1.2. Antigen detection

Immunofluorescence and IP procedures for diagnosis are generally applied to suspect isolates rather than directly to infected exudates or tissues. This approach is preferred because the organisms mycoplasmas are too small to recognise conclusively-be reliably identified under the a light microscope, and because corresponding-suitable negative and positive control samples for direct tissue testing are unlikely to be readily available.

1.2.1. Indirect fluorescent antibody test

The recommended technique for the IFA test (Rosendal & Black, 1972) requires an agar-culture on agar of the unknown isolate, consisting of numerous small discrete colonies, a known MG or MS culture as a-positive control, and a culture of another mycoplasma species, such as *M. gallinaceum* or *M. gallinarum* as a-negative control. Also required are polyclonal rabbit anti-MG or MS serum, a normal rabbit serum and an anti-rabbit immunoglobulin fluorochrome-conjugated serum. Sera may be prepared in species other than rabbits, but monoclonal antibodies (MAbs) should not be used because MG or MS demonstrate variable expression of their surface

epitopes and an MAb may fail to recognise the target microorganism. Suitable working dilutions in sterile phosphate buffered saline (PBS; 0.01 M, pH 7.2) of the anti-MG or MS serum and the conjugate are first determined by cross-titration, and are selected for use at two-to-four-fold dilutions less than the actual end-points. These are applied to the unknown mycoplasma colonies that have been previously grown on agar plates as indicated below.

1.2.1.1. Test procedure

- i) From colony-bearing agar plates, cut blocks of about 1.0 × 0.5 cm and place them on to labelled microscope slides with the colonies uppermost.
- ii) To make subsequent orientation possible, cut off the lower right-hand corner of the blocks. One block with the unknown isolate, a block with the known MG culture, a block with the known MS culture and a block with a different but known mycoplasma culture are placed on one slide. A block of the unknown isolate is placed on another slide.
- iii) Add a drop of suitably diluted MG (or MS) antiserum to the surface of each block of the first slide and add normal rabbit serum to the single block on the second slide.
- iv) Incubate all blocks for 30 minutes at room temperature in a humid atmosphere.
- v) Place each block in a labelled tube containing PBS, pH 7.2 and wash gently for 10 minutes on a rotary mixer, then rewash as before, and finally return the blocks to the original microscope slides.
- vi) Blot excess moisture from the sides of the blocks. Add one drop of the diluted conjugate to each block, and incubate and wash as before.
- vii) Return the blocks to their original slides, and examine the colonies by incident light using fluorescence microscopy.

Interpretation of the results is subjective and requires some expertise; comparisons with the controls must give the correct reactions and are essential.

Some laboratories use fluorescein-conjugated antiserum in a direct fluorescent antibody test (DFA). A technique that is widely used for DFA is one in which the reagents are applied successively within stainless steel cylinders placed on the original mycoplasma agar plate. Although this is quick and easy to perform, the results obtained are less specific than using the indirect method, which is therefore preferred.

1.2.2. Indirect immunoperoxidase/immunobinding test

The indirect IP test follows a similar principle to the IFA test except that the binding of specific antibodies to colonies *in situ* is detected by adding an anti-rabbit immunoglobulin that has been conjugated to the enzyme peroxidase. A positive reaction is then visualised by adding an appropriate substrate which, on oxidation, produces coloured colonies. An immunobinding procedure can also be used in which the test colonies are blotted on to nitrocellulose (Kotani & McGarrity, 1985) and then reacted in a similar manner. As with IFA, polyclonal sera should be used for serotyping isolates by IP. The advantage of the IP test over immunofluorescence is that the IP test does not require an expensive fluorescence microscope.

1.2.3. Growth inhibition test

In the GI test, the growth of mycoplasmas is inhibited by specific antiserum, enabling species to be identified. It is relatively insensitive and sera must be high-titred, monospecific and prepared in mammalian hosts as poultry sera do not always inhibit mycoplasma growth efficiently. The organism under test must be in pure culture (cloned) and several dilutions should be tested; a concentration of 10⁴ colony-forming units (CFU/ml) is optimal. The rate of growth of the organism may influence growth inhibition, and it is helpful to retard growth initially by incubating at 27°C for 24 hours, followed by incubation at 37°C thereafter. Details of the test and its interpretation are published elsewhere (Clyde, 1983).

1.3. Molecular methods – detection of nucleic acids

PCR assays are routinely used in many laboratories and are characterised by good sensitivity. These methods represent a good alternative to *in-vitro* culture of mycoplasmas because they are based on the detection of specific DNA sequences of the pathogen directly on clinical samples or isolates grown *in vitro*. MG or MS DNA ~~are~~^{is} amplified by PCR using species-specific primers. The real-time PCR with fluorescent labelled probes is becoming increasingly used, shortening the detection time compared with conventional PCR. Great care has to be taken to avoid contamination of samples with MG or MS DNA

from nearby necropsy rooms, mycoplasma cultures ~~laboratories~~ or from previous PCR runs (see Chapter 2.1.2 *Biotechnology advances in the diagnosis of infectious diseases* and Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*). ~~Several Different~~ commercial kits for PCR and real-time PCR of both MG and MS are available, and several in-house procedures have also been published (Dijkman *et al.*, 2017; Raviv & Kleven, 2009).

A conventional PCR and a real-time PCR for MG and MS are described below. Within the PCR-based methods, the denaturing gradient gel electrophoresis (DGGE) technique ~~could~~ can be applied for the identification of most avian mycoplasmas, including MG and MS, but this test is validated only on isolates from mycoplasma culture. This method is described in Section B.1.3.2.

Genotyping techniques based on the analysis of the *mgc2*, *pvpA* and *vlhA* genes are currently widely applied for the classification of MG (Armour & Ferguson-Noel, 2015; García *et al.*, 2005) and MS isolates, respectively (Hammond *et al.*, 2008). Moreover, a core genome multilocus sequence typing (MLST) scheme for *M. gallisepticum* and two *M. synoviae* MLST schemes have been published (~~Dijkman *et al.*, 2016~~; El-Gazzar *et al.*, 2017; Ghanem *et al.*, 2017) and are likely to become globally applied epidemiological tools.

Extraction of nucleic acids

Different kits and procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid is important and can be checked using a suitable method as appropriate to the circumstances.

1.3.1. Conventional polymerase chain reaction

The assay described is a validated PCR for MG and MS detection based on a 16S rRNA fragment amplification (Lauerman, 1998). Another widely used method based on *mgc2* gene for MG detection (García *et al.*, 2005) is reported. It must be remembered that unrelated strains may occasionally share DNA sequences and give DNA amplification bands in different laboratory conditions. All new PCRs require validation using criterion in Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals* and Chapter 2.2.3 *Development and optimisation of nucleic acid detection assays*. The conventional PCR for MS reported in this chapter can also detect *M. bovirhinis* DNA. For laboratories monitoring MG using the *mgc2*-PCR endpoint protocol it is advised to also consider implementing the real-time PCR protocol described below. This recommendation is based on the possibility of mutations occurring at the primer attachment site, which may hinder detection of atypical MG strains (Matucci *et al.*, 2023). Having both PCR protocols available in the laboratory enhances the ability to identify a broader range of MG variants.

1.3.1.1. DNA extraction

~~DNA is extracted from swab samples (up to 10 swabs may be pooled) suspended in PCR-grade PBS in a 1.5 ml snap-cap Eppendorf tube. Several commercial extraction kits based on the spin column are available for DNA extraction from swabs, tissues, etc. Automated extraction of Mycoplasma DNA is possible with specific commercial kits. The appropriate kit for the type of sample should be selected and the manufacturer's protocol for DNA extraction should be followed.~~

<u>Pathogen/ target gene</u>	<u>Primer (5'–3')</u>	<u>Concentration</u>	<u>Cycling parameters</u>
<u>Method 1: Lauerman, 1998; GenBank Accession No.: M22441; amplicon size: 183 bp</u>			
<u>16S rRNA for MG</u>	<u>MG-14F: GAG-CTA-ATC-TGT-AAA-GTT-GGT-C</u> <u>MG-13R: GCT-TCC-TTG-CGG-TTA-GCA-AC</u>	<u>0.2 µM</u> <u>0.2 µM</u>	<u>1 cycle of 95°C/10 min; 40 cycles of:</u> <u>94°C/30 sec, 55°C/30 sec and</u> <u>72°C/60 sec, 1 cycle of 72°C/5 min</u>
<u>Method 2: García <i>et al.</i>, 2005; GenBank Accession No.: U34842; amplicon size: 236–302 bp</u>			
<u><i>mgc2</i> for MG</u>	<u>MG-1: CGC-AAT-TTG-GTC-CTN-ATC-CCC-AAC-A</u> <u>MG-2: TAA-ACC-CRC-CTC-CAG-CTT-TAT-TTC-C</u>	<u>0.4 µM</u> <u>0.4 µM</u>	<u>1 cycle of 95°C/10 min; 40 cycles of:</u> <u>94°C/30 sec, 55°C/30 sec and</u> <u>72°C/60 sec, 1 cycle of 72°C/5 min</u>
<u>Method 3: Lauerman, 1998; GenBank Accession No.: X52083; amplicon size: 211 bp</u>			
<u>16S rRNA for MS</u>	<u>MS-F: GAG-AAG-CAA-AAT-AGT-GAT-ATC-A</u> <u>MS-R: CAG-TCG-TCT-CCG-AAG-TTA-ACA-A</u>	<u>0.4 µM</u> <u>0.4 µM</u>	<u>1 cycle of 95°C/10 min;</u> <u>40 cycles of: 94°C/30 sec,</u>

			55°C/30 sec and 72°C/60 sec, 1 cycle of 72°C/5 min

1.3.1.2. Primers

PCR method	PCR primer sequences	Expected amplicon
16S rRNA for MG	MG-14F: 5'-GAG-CTA-ATC-TGT-AAA-GTT-GGT-C-3'	183 bp
	MG-13R: 5'-GCT-TGG-TTG-CGG-TTA-GCA-AC-3'	
<i>mgc2</i> for MG	MG-1: 5'-CGC-AAT-TTG-GTC-CTN-ATC-CCC-AAG-A-3'	236-302 bp
	MG-2: 5'-TAA-ACC-CRC-CTC-CAG-CTT-TAT-TTC-C-3'	
16S rRNA for MS	MS-F: 5'-GAG-AAG-CAA-AAT-AGT-GAT-ATC-A-3'	211 bp
	MS-R: 5'-CAG-TGG-TCT-CCG-AAG-TTA-ACA-A-3'	

1.3.1.3. Polymerase chain reaction

- The reaction mixture should be prepared, according to the manufacturer's instruction, in a separate clean area using a set of dedicated pipettes. For each sample, dispense into a PCR tube 45 µl volume of mix containing 0.4 µM of each primer for 16S rRNA MS and *mgc2* MG, or 0.2 µM for 16S rRNA MG. An internal amplification control (IAC) could be included as a commercial exogenous kit or using designed primers for endogenous sequence (e.g. 18S rRNA for eukaryotic derived samples) amplifying at same PCR conditions. The reaction mixture should be overlaid with a few drops of light weight mineral oil unless the thermocycler is equipped with a heated lid. The tubes are taken to another clean area where the appropriate extracted DNA sample (5 µl) is added to each tube. Positive and negative control DNA should be run in each assay. The tubes are then placed in a thermal cycler for the following cycles: 40 cycles: 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 60 seconds, 1 cycle (final extension): 72°C for 5 minutes and soak at 4°C.
- For amplification of 16s rRNA MS and *mgc2* MG using a hot start Taq polymerase, run the thermocycler at the following profile:

Polymerase activation	95°C	10 minutes	
40 cycles	95°C	45 seconds	
	54°C	60 seconds	
	72°C	60 seconds	
1 cycle (final extension)	72°C	7 minutes	soak at 4°C

1.3.1.4. Electrophoresis

PCR amplification products are detected by conventional gel electrophoresis, incorporating appropriate size markers. The stained products are visualised under UV light or with silver nitrate under a hazardous chemical hood. Alternatively, the amplified product can be run in a capillary electrophoresis machine loaded with appropriate size markers. The base-pair dimension of the amplified fragments could be statistically different by about 10% than expected using this method. Examination of the PCR products should be carried out in a laboratory area, well separated from places where other steps in the PCR procedure are performed.

1.3.2. 16s-rDNA-PCR and denaturing gradient gel electrophoresis (DGGE)

The 16S-rDNA-PCR-DGGE technique is a method that can be used for the identification of mycoplasma isolates (McAuliffe *et al.*, 2005), including avian mycoplasmas, from cultivated cultured broths, or agar colonies. It can also be used on or DNA extracted from clinical specimens. The genetic target is the V3 region of the 16S gene, which is amplified by the combination of using a mycoplasma-specific primer (reverse primer) and a universal bacterial one primer (forward primer) containing a GC-clamp (40 repeated GC).

The method is based on the migration of DNA fragments in the gel following strand separation caused by chemical denaturants in the gel. It is capable of detecting-resolving single-base mutations in DNA. After migration on the denaturing gel, the pattern of the band(s) produced by

the unknown samples is compared with ~~positive that of known~~ avian mycoplasma controls, ~~which are run in parallel. This technique is capable of detecting~~ enables the detection of both single and mixed avian-Mycoplasma spp., infection and co-infections, within a single sample (Catania *et al.*, 2014; 2016b).

Pathogen/ target gene	Primer (5'–3')	Concentration	Cycling parameters
Method 1: McAuliffe <i>et al.</i> , 2005; GenBank Accession No.: not available, amplicon size: 340 bp			
<u>16S rDNA V3 region</u>	<u>R543: 5'-ACC-TAT-GTA-TTA-CCG-CG</u> <u>GC 341 F: 5'-CGC-CCG-CCG-CGC-GCG-GCG-GGC-</u> <u>GGG-GCG-GGG-GCA-CGG-GGG-GCC-TAC-GGG-</u> <u>AGG-CAG-CAG-3'</u>	<u>0.5 µM</u> <u>0.5 µM</u>	<u>1 cycle of 95°C/5 min;</u> <u>35 cycles of: 95°C/60 sec,</u> <u>58°C/45 sec and 72°C/60 sec.</u> <u>1 cycle of 72°C/20 min</u>

i) Primers

The following primers are used:

~~R543: 5' ACC TAT GTA TTA CCG CG 3~~
~~GC 341 F: 5' CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG 3'~~

ii) Polymerase chain reaction

The PCR reaction mixture should be prepared in a separate clean area as follows (final volume: 25 µl):

H ₂ O Ultra-pure	12.5 µl
5 × PCR buffer	5.00 µl
dNTP (10 mM)	1.00 µl
F Primer (50 µM)	0.25 µl
R Primer (50 µM)	0.25 µl
Taq (5 U/µl)	0.25 µl
DMSO (1%)	0.25 µl
MgCl ₂ (25 mM)	4.00 µl

A 23.5 µl master mix is dispensed into the tubes and 1.5 µl of nuclease free water/sample/control DNA is then added.

Tubes are then placed in a thermocycler at the following profile:

1 cycle (initial denaturation)	95°C	5 minutes	
35 cycles:	95°C	1 minute	
	58°C	45 seconds	
	72°C	60 seconds	
1 cycle (final extension)	72°C	20 minutes	soak at 4°C

iii) Electrophoresis

20 µl of each PCR product is loaded in 10% polyacrylamide/bis (37.5:1) gels, with denaturing gradients from 30 to 60% (where 100% is 7 M urea and 40% [v/v] deionised formamide) in 1× TAE buffer. The electrophoresis is performed at 100 V, 300 mA at a temperature of 60°C for 18 hours, the run time could change based on the DGGE device and on the gel dimension. Gels are then stained with a suitable DNA stain for 30 minutes (5 µl in 50 ml of 1× TAE buffer) and visualised under UV illumination.

1.3.3. Real-time PCR

Species-specific real-time PCRs were developed to increase the throughput of diagnostic samples (Raviv & Kleven, 2009). This method uses specific fluorescent probes that increase the cost of specific analysis, but avoid potential contamination post-amplification. MG detection by real-time PCR targets the *mgc2* gene, while MS detection targets the 16S–23S ISR. The assay is conducted as a duplex singleplex amplification that for each species, and it may includes an a commercial or in-house internal amplification control (IAC). A detection limit of 10 copies of MS DNA per reaction and 1 copy of MG DNA is determined.

Pathogen/ target gene	Primer/probe (5'–3')	Concentration	Cycling parameters
Method 1: Raviv & Kleven, 2009; GenBank Accession No.: AE015450			
MG	MGFr: TTG-GGT-TTA-GGG-ATT-GGG-ATT MGRr: CCA-AGG-GAT-TCA-ACC-ATC MGPr: Texas Red-TGA-TGA-TCC-AAG-AAC-GTG-AAG-AAC-ACC-BHQ1	0.5 µM 0.5 µM 0.2 µM	1 cycle 95°C/10 min 45 cycles of: 95°C/15 sec and 60°C/30 sec
Method 2: Raviv & Kleven, 2009; GenBank Accession No.:			
MS	MSFr: CCT-CCT-TTC-TTA-CGG-AGT-ACA MGRr: CTA-AAT-ACA-ATA-GCC-CAA-GGC-AA MGPr: FAM-ATT-CTA-AAA-GCG-GTT-GTG-TAT-CGC-T-BHQ1	0.5 µM 0.5 µM 0.2 µM	1 cycle 95°C/10 min 45 cycles of: 95°C/15 sec and 60°C/30 sec

i) — Primers

For	Primers and probes
MG	MGFr F 5'-TTG-GGT-TTA-GGG-ATT-GGG-ATT-3'
	MGRr 5'-CCA-AGG-GAT-TCA-ACC-ATC-3'
	MGPr 5'-Texas Red-TGA-TGA-TCC-AAG-AAC-GTG-AAG-AAC-ACC-BHQ1-3'
MS	MSFr 5'-CCT-CCT-TTC-TTA-CGG-AGT-ACA-3'
	MSRr 5'-CTA-AAT-ACA-ATA-GCC-CAA-GGC-AA-3'
	MSPr 5'-FAM-ATT-CTA-AAA-GCG-GTT-GTG-TAT-CGC-T-BHQ1-3

A commercial IAC DNA kit could be included into the amplification mix to reduce the likelihood of false negatives results.

ii) Polymerase chain reaction

The amplification is conducted in a 96-well real-time thermocycler. A tube reaction is prepared for each sample to be tested for MG or MS. Each reaction contains a total of 25 µl comprising 5 µl of target DNA, 12.5 µl of 2× of universal real-time PCR master mix, 5 µM of each final primer and 0.2 µM final probe. An IAC reagent could be added in each reaction following manufacturer's instructions.

A common amplification protocol is used:

1 cycle	95°C	10 minutes	(single denaturation step)
45 cycles:	95°C	15 seconds	
	60°C	30 seconds	

Fluorescence signal for specific *Mycoplasma* probe and IAC is acquired in the appropriate detector channel during the extension step.

The cycle threshold value (Ct = Cq quantification cycle) automatically calculated by the software should be used. Cq values of 35 or lower are considered as positive, IAC should amplify in negative samples with Cq between 30 and 40, otherwise it could be set as inhibited.

1.3.4. Molecular typing

Different molecular methods are also available for ~~differentiation of~~ differentiating MG and MS strains; ~~but however,~~ their use ~~tends to be restricted at present~~ is currently limited to specialised laboratories. Sequence-based methods have been developed to identify circulating strains, ~~enhance understanding of mycoplasma~~ to understand better the epidemiology of mycoplasma ~~and to support control measures,~~ which in turn helps refine and improve control strategies. MS strains can be identified and classified with methods such as MLST (Dijkman *et al.*, 2016; El-Gazzar *et al.*, 2017), sequencing of *vha* 5' and number of proline-rich repeats (Hammond *et al.*, 2008), differentiation of MS-H with *obg* and *oppF*-1 mutations (Shahid *et al.*, 2013; Zhu *et al.*, 2017). Vaccine strains can be differentiated with DIVA (detection of infection in vaccinated animals) analysis (Dijkman *et al.*, 2017). MG and MS strains can be discriminated using core genome MLST (Ghanem *et al.*, 2017; Ghanem & El-Gazzar, 2018). Novel approaches such as

MLVA (multilocus variable tandem array; Kreizinger *et al.*, 2018) and MAMA (mismatch amplification mutation assay) are very promising for discriminating field isolates from vaccine strains, but are not yet widely available.

These newer methods are rapidly replacing the other molecular typing techniques, such as restriction endonuclease analysis, pulsed-field gel electrophoresis, amplified fragment length polymorphism and random amplified polymorphic DNA analysis because they are too-less labour intensive and costly-more cost-effective for large-scale typing. Based on the high speed of improvement of the new analytical methods as well as the greater availability of increasingly sophisticated equipment, the methods currently available may be replaced in a short time. Furthermore, the nomenclature of avian mycoplasmas strains nomenclature shall is expected to be reviewed in the near future for-to enable a more usable-practical classification of circulating mycoplasma strains and better-to enhance the effectiveness of disease control policies.

2. Serological tests

Commonly used The serological tests for MG and MS often in common use lack adequate specificity and/or sensitivity. Therefore, it is strongly recommended that their use is limited to flock-level monitoring rather than testing individual birds. Diagnosticians wishing-intending to use such-these tests are advised to establish the test should evaluate their sensitivity and specificity (Chapter 1.1.6) under their own laboratory conditions. It should also be noted that these tests have not been validated for use with sera from day-old birds or from game birds (Bradbury, 2005).

The most commonly used serological tests are rapid serum agglutination (RSA), enzyme-linked immunosorbent assay (ELISA) and haemagglutination inhibition (HI). Several others have been described. Other methods, such as radioimmunoassay, micro-immunofluorescence, immunoblotting (Welchman *et al.*, 2013) and IP assays, have been described but are rarely applied in routine diagnostics-used. The number of sera to be tested within a flock depends on the desired level-of-detection threshold and the confidence levels-limits required. Minimal testing requirements, including frequency of testing for international trade within the European Union, have been described, for example for MG in Council Directive 2009/158/EC. Minimal requirements and approved tests are also set out for members of the National Poultry Improvement Plan (NPIP) of the USA.

Poultry companies that routinely use using-ELISA for large-scale screening large numbers of blood sera for viral virus-antibodies may find this type of assay convenient also for mycoplasma testing. In addition to blood serum, egg yolk can also be used for serological screening of layer hens via ELISA testing. In fact, IgY antibodies are selectively transferred from the hen's bloodstream into the egg yolk, whereas IgM and IgA are secreted into the egg albumen and are not transferred into the embryonic circulation. Chicks absorb only about 10% of the maternal IgY present in the egg yolk, resulting in approximately an eight-fold lower concentration of antigen-specific antibodies in neonatal serum, which declines after 2 days post-hatch. Endogenous production of IgY, IgM, and IgA in chicks typically becomes detectable around 10 days after hatching (Härtle *et al.*, 2022). Therefore, any serological screening conducted using blood collected before this period should be considered a screening of the layer hens rather than the chicks. The ELISA will not be described in detail here because several MG and MS kits are available commercially. Instead, emphasis is placed on the HI test, for which commercially available reagents are limited.

2.1. Rapid serum agglutination test

Sera are collected from a sample of the flock and, if not tested immediately, are stored at 4°C and not frozen. The test should be carried out at room temperature (20–25°C) within 72 hours of serum collection using reagents kept at room temperature. Prior centrifugation of sera will reduce nonspecific reactions. The RSA kits are available commercially, but they may vary in specificity and sensitivity from different manufacturers and from batch to batch. They must be stored according to the manufacturer's instructions. Suitable RSA-stained antigens may also be prepared 'in-house' using culture methods as described in Section B.1.; these are then stained with crystal violet dye. Quality control standards for mycoplasma antigens for serological tests are described below.

2.1.1. Test procedure (Allan & Gough, 1974)

- i) Drop one volume (approximately 0.02 ml) of serum on to a clean white tile or glass plate followed by one volume of stained MG or MS antigen. Do not allow the serum to dry out before addition of the antigen. It is important to shake the antigen bottle vigorously and frequently during use to keep the correct amount of antigen in suspension.
- ii) Use a stirring rod to spread the mixture over a circular area of approximately 1.5 cm diameter. Rock the tile or plate for 2 minutes. Agglutination is indicated by flocculation of the antigen within 2 minutes.
- iii) Include known positive and negative controls ~~in the test.~~

-
- iv) Retest serial dilutions of any sera that agglutinate after heating at 56°C for 30 minutes. If they still react strongly, they are considered to be positive on dilution (1/4 or more).

In the USA, MG and MS positive reference antisera can be obtained from the USDA National Veterinary Services Laboratories (NVSL), and in Europe from Anses Ploufragan¹, France. MG, MS and control sera produced in chickens or in turkeys, with a range of titres, can be purchased. Sets of antisera can be purchased also from the Department of Avian Medicine, University of Georgia, subject to availability.

There are no international standards for interpreting these tests, but a high proportion of positive sera in a flock (10% or more) suggests MG infection, especially if confirmed by HI test or ELISA. For further confirmation, the flock should be retested within a month. Inconclusive results make it necessary to attempt to isolate the organism or to demonstrate the presence of its DNA. Doubtful results for MG or MS should be investigated by performing tests with MS antigen (and *vice versa*) as infection with these organisms sometimes causes cross-reactions.

Tests can be conducted on yolk as well as sera although the yolk must first be diluted or extracted.

2.2. Haemagglutination inhibition test

MG and MS are capable of haemagglutinating avian red blood cells (RBCs), which can be inhibited by specific antibodies in sera. A strain that grows well and reliably haemagglutinates should be selected. The HI test requires a satisfactory haemagglutinating MG and MS antigens, washed fresh chicken or turkey RBCs, as appropriate, and the test sera. The antigen can be either a fresh broth culture or a concentrated washed suspension of the mycoplasma cells in PBS. It may be difficult to sustain a supply of high-titred broth culture antigen; however, the use of concentrated antigen (usually containing 25–50% glycerol and stored at –70°C), increases the likelihood of nonspecific reactions. In the USA, MG and MS haemagglutination (HA) antigens can be purchased from the NVSL.

The HI test follows well-known procedures (Allan & Gough, 1974). The HA titre of the antigen is first determined in doubling dilutions, the HA unit being defined as the least amount of antigen giving complete HA in the test system employed. The HI test should be performed using 4 HA units by the following method or a method having equivalent sensitivity as determined by tests with known positive sera.

All HA titrations and HI tests are best performed in multiwell plastic plates with V-shaped wells and using constant volumes of 50 µl. A positive and a negative control serum are incorporated into each test. One row of eight wells is required for each serum under test.

2.2.1. Test procedure

- i) Add 50 µl of PBS to the first well in each row.
- ii) Add 8 HA units of antigen in 50 µl volumes to the second well in each row and add 50 µl of 4 HA units of antigen to each of wells 3 to 8.
- iii) Add 50 µl of a previously-prepared 1/5 dilution of the serum under test to the first well, mix, and transfer 50 µl to the second well, and so on, and discard 50 µl from the last well. The first well is the serum control well.
- iv) Six wells are required for the antigen control. Add 50 µl of PBS to wells 2 to 6, inclusive, and add 50 µl of the 8 HA unit antigen to wells 1 and 2. Mix the contents of well 2 and transfer 50 µl to well 3, mix and repeat up to well 6, and discard 50 µl.
- v) Two wells are required for the RBC control. Add 50 µl of PBS to each of these.
- vi) Add 50 µl of a 0.5% suspension of RBCs (chicken cells for chicken serum and turkey for turkey serum) to all wells.
- vii) Shake the plate lightly to ensure thorough mixing of the well contents, and read after standing for approximately 50 minutes at room temperature or when the antigen titration is reading 4 HA units. For reading, the plate should be tilted and only those wells in which the RBCs 'stream' at the same time as those in the RBC control wells should be considered to be inhibited. The serum control should show a clear button of RBCs and the positive and negative controls should react as expected. The HI titre is the highest serum dilution exhibiting complete inhibition of HA.

¹ Agence Nationale de Sécurité Sanitaire de l'Alimentation, de l'Environnement et du Travail (Anses) Ploufragan, Mycoplasma Bacteriology Unit, 22440 Ploufragan, France.

Sera giving nonspecific HA must be adsorbed to remove all nonspecific haemagglutinins so that a clear button is obtained in the control well without HA antigen. The adsorption is carried out by incubating 1 ml of the serum dilution with 6–8 drops of packed washed chicken or turkey RBCs. The cells are removed after incubation at 37°C for 10 minutes, and the supernatant is tested for haemagglutinating activity.

There is no recognised definition of positive and negative results for international trade.

2.3. Enzyme-linked immunosorbent assay

Several commercial MG and MS antibody ELISA kits are marketed and widely used in diagnostic laboratories. These ELISAs use different cut-offs and mathematical formulas to convert the ELISA result into a titre value. This means that every ELISA has its own interpretation and the titre results of different ELISAs on the same serum could differ. Apart from blood serum, the egg yolk can also be used to serologically screen layer hens through ELISA (Härtle *et al.*, 2022).

2.4. Quality control of *Mycoplasma gallisepticum* and *M. synoviae* antigens

2.4.1 *Mycoplasma gallisepticum* antigens

Antigens are usually prepared from the S6 strain or the A5969 strain of MG. Antigens prepared from other strains may also be used when necessary.

i) MG antigen for the RSA test

The methods of quality control described below apply solely to suspensions of MG stained with a suitable dye containing preservative and intended for use in the rapid plate agglutination test with serum. Such antigens are available commercially.

On microscopic examination, the antigen should appear as a homogeneous suspension without floccules or precipitates and the suspending liquid should be free from residual dye. It must be sterile with a pH of between 6.5 and 7.0 and stored at 5±3°C. It must be warmed to room temperature before use.

The sensitivity and specificity of the antigen is determined with respect to its reaction with known positive sera of high and low titre and known negative sera. A positive reaction is recognised by the formation of coloured floccules and the clearing of the suspending medium. The criteria described above must continue to apply until the expiry date declared by the manufacturer.

ii) MG antigen for the HI test

The test is preferably performed with live, actively growing cultures. The antigen must be free from contamination with bacteria and fungi.

iii) MG antigen for the ELISA

It may be difficult to prepare satisfactory antigen for use in the indirect ELISA without considerable prior experimentation and confirmation of sensitivity and specificity. Use of a validated commercial kit is the best approach for most diagnostic laboratories.

2.4.2. *Mycoplasma synoviae* antigens

Antigens prepared from the WVU 1853 strain or other suitable strains should be used.

i) MS antigen for the RSA test

The specifications apply as for MG antigen for the RSA test.

ii) MS antigen for the HI test

The same specifications apply as for MG antigen for the HI test.

2.4.3. Additional comments

Sera giving nonspecific reactions to the RSA test do not usually give a positive reaction in the HI test using live HA antigen. Positive RSA reactions can be confirmed by the HI test with sera taken after the first 2–3 weeks of infection (the time taken for HI antibodies to develop). However, the

HI test tends to be strain specific (Kleven *et al.*, 1988) and therefore may lack sensitivity. ELISA may be a useful alternative.

Samples of serum should not be frozen before use in RSA tests. They should be free from haemolysis and contamination to avoid nonspecific reactions. The use of inactivated vaccines for other diseases may result in nonspecific reactions. Samples should be tested as soon as possible (within 72 hours) because mycoplasma antibodies may deteriorate on storage. Sera may be inactivated in a water bath at 56°C for 30 minutes.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

The preferred method of control is to maintain MG- and MS-free flocks, to increase the biosecurity measures and to avoid or to limit mycoplasma production by the infected breeder groups, containing the spread of these pathogens through vertical transmission (Kleven, 2008; Ferguson *et al.*, 2020). Vaccination should be considered only in situations where field exposure is inevitable, or in specific cases, such as on multi-age sites. Potential exposure of neighbouring poultry flocks should also be carefully considered.

Two types of vaccines, live and killed, are available for the control of MG and MS: mild to avirulent live MG or MS strains and inactivated oil-emulsion bacterins. Several scientific papers have been published on this issue, providing evidence of the effect of vaccination on reducing drop of egg production, on limiting respiratory signs, airsacculitis, and in reducing egg transmission.

Although antigenic variability is present among MG or MS strains, it is thought that vaccination with a single MG or MS strain is sufficient to achieve a good level of protection against the homologous species. Live vaccines can be considered a good and effective tool for the management and containment of mycoplasmoses. However, until recently, it was not easy to distinguish the vaccine strain from the wild strains using the available tests (Catania, 2016b). However, the newer biomolecular techniques, while not simple or easy, have been validated for differentiating the strains (Dijkman *et al.*, 2017; Kreizinger *et al.*, 2017), though they are usually based on single-point mutations.

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.

Bacterin-based vaccines are used to prepare the immune system for exposure to the disease to reduce the clinical signs, such as egg production drops that occur as a consequence of MG infection in layers. Their use at present is limited mainly to MG.

The use of live vaccines is equivalent to a 'controlled exposure'. The objective is to infect the flock with a mild, immunogenic MG or MS strain at an age when little or no significant damage occurs. Such exposure results in resistance to challenge later in life, particularly on multi-age commercial sites. Successfully vaccinated birds should be resistant to respiratory disease, airsacculitis, egg production drops or other specific lesions caused by MG or MS. Vaccination should also reduce transmission of the pathogen through the egg in breeders.

1. Outline of production and minimum requirements for vaccines

1.1. Characteristics of the seed

1.1.1. Live vaccine

The vaccine strain should be immunogenic, must readily colonise the upper respiratory tract, and cause minimal damage to the respiratory system. A strong antibody response does not necessarily correlate with immunity.

The seed culture should be free from all extraneous agents. The culture should be cloned to ensure purity. If desired, restriction endonuclease patterns of the mycoplasmal DNA, or other methods such as 16s PCR DGGE, can be run to be sure of the identity and purity of the strain.

The seed culture should be stable with no tendency to revert to virulence. This can be confirmed with ten back passages in susceptible chickens. Contact chickens can be introduced at weekly intervals. If necessary, tracheal swabs can be taken from infected chickens and can then be inserted into the trachea of contact chickens. Transmission of the organism should be proven. The resulting isolate can then be used to challenge susceptible chickens.

1.1.2. Killed vaccine

For killed vaccines the most important characteristics are high yield and good antigenicity. It is assumed, but not proven, that virulent strains are desirable. The seed culture should be free from all extraneous organisms.

1.2. Method of culture

The seed culture may be propagated in a medium similar to that described above (Section B.1) for live vaccines, the broth culture is lyophilised or frozen at -70°C or colder. For bacterins the culture must be concentrated and resuspended in a small volume of saline or PBS before the emulsion is prepared.

1.3. Validation as a vaccine

Data on efficacy should be obtained before bulk manufacture of vaccine begins. Chickens should be vaccinated by the same route that will be used in the field. Vaccinated birds should be challenged, and protection should be determined against respiratory signs, nasal discharge, and/or airsacculitis. Ideally, protection against egg-production losses should be evaluated, but such challenge trials are expensive and cumbersome.

Efficacy test: Groups of 20 specific pathogen free (SPF) chickens or at least mycoplasma-free chickens, 2 weeks of age or older, are vaccinated by eyedrop or other route of administration with one field dose of live vaccine, or subcutaneously or intramuscularly with one dose (usually 0.5 ml) of bacterin. A similar group of unvaccinated chickens is maintained separately as controls. All chickens should be challenged with a 24-hour broth culture of a virulent strain of MG, 2–3 weeks post-vaccination. A simple challenge method is inoculation of 0.1 ml of the challenge culture into the posterior thoracic air sac. All birds are necropsied 7–10 days post-challenge, and air sac lesions are scored. Alternative methods are to challenge by inoculating 0.1 ml into the infraorbital sinus and examining the birds for nasal discharge from 7 to 14 days post-challenge or to challenge by aerosol and measure the thickness of the tracheal mucosa on microscopic sections at four to six equidistant predetermined points (Whithear, 1996).

1.4. Method of manufacture

1.4.1. Procedure

The vaccine must be manufactured in suitable clean and secure accommodation, well separated from diagnostic facilities or commercial poultry. Special care must be taken to avoid MG contamination of other products manufactured in the same facility.

Production of vaccine should be on a seed-lot system, using a suitable MG strain of known origin, passage history, and purity. The growth medium is similar to that given above. The serum used in the growth medium should be inactivated at 56°C for 1 hour to prevent contamination with any mycoplasma that may be present, and filter sterilised. A source of SPF serum is desirable.

Broth medium is inoculated, with a rapidly growing inoculum, at a rate of approximately 5% (v/v). Incubation is at 37°C . Production can be in batches using large flasks or in a fermenter. In batch cultures, harvest is approximately 24 hours after inoculation. Live vaccines are preserved by lyophilisation or by freezing at -70°C , in liquid nitrogen, or on dry ice.

For bacterin production, the antigen must be concentrated, usually by centrifugation, ultrafiltration, or other suitable method. Bacterins are made as water-in-oil emulsions, typically 80% mineral oil, 20% aqueous, with suitable emulsifying agents.

1.4.2. Requirements for ingredients

See Chapter 1.1.8 with special focus on products of biological origin originating from a country with negligible risk for transmissible spongiform encephalopathies.

1.4.3. In-process control

i) Antigen content

At harvest, the titre should be from 10^8 to 10^9 CFU/ml. The antigen concentration of bacterins is difficult to standardise but may be based on packed cell volume, which is typically 1% (v/v) packed cells in the final product.

ii) Inactivation of killed vaccines

Inactivation is frequently done with either beta-propiolactone or formaldehyde. The inactivating agent and the inactivation procedure must be shown under the conditions of vaccine manufacture to inactivate the vaccine organism and potential contaminants.

Prior to inactivation, care should be taken to ensure a homogeneous suspension free from particles that may not be penetrated by the inactivating agent. A test for inactivation should be carried out by culture in mycoplasma broth on each batch of both the bulk harvest after inactivation and the final product. No evidence of growth of mycoplasma should be observed.

iii) Sterility of killed vaccines

Oil used in the vaccine must be sterilised by heating at 160°C for 1 hour, or by filtration, and the procedure must be shown to be effective. Tests appropriate to oil-emulsion vaccines are carried out on each batch of final vaccine as described, for example, in the British Pharmacopoeia (Veterinary) 1985.

1.4.4. Batch control

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) Safety

a) **Live vaccine safety test**

The birds vaccinated in the efficacy test given above can be used to evaluate the safety of the vaccine.

b) **Killed vaccine safety test**

Birds vaccinated in the efficacy test described above may be observed for adverse local or systemic effects.

iii) Batch potency

Potency tests for both live and killed vaccine can be conducted by the procedures given above for the efficacy test. The titre of live vaccines should be sufficient to induce infection by the route recommended by producers per dose per bird to last until the expiry date.

iv) Stability

Evidence should be provided on three batches of vaccine to show that the vaccine passes the batch potency test at 3 months beyond the requested shelf life.

1.5. Requirements for regulatory approval

1.5.1. Manufacturing process

For vaccine approval, all relevant details concerning manufacture of the vaccine and quality control testing (see Section C.2.1 and 2) should be submitted to the Authorities. Information should be provided from three consecutive vaccine batches to demonstrate consistency of production.

1.5.2. Safety requirements

i) Precautions (hazards)

Oil-emulsion vaccines cause serious injury to the vaccinator if accidentally injected into the hand or other tissues. In the event of such an accident, the person should go at once to a hospital, taking the vaccine package with him or her. Each vaccine bottle and package should be clearly marked with a warning of the serious consequences of accidental self-injection. Such wounds should be treated by the casualty doctor as a 'grease gun injury'.

Personnel vaccinating birds with live virus vaccines by aerosol spray should wear protective clothes and masks.

1.5.3. Efficacy requirements

To register a commercial vaccine, a batch or batches produced according to the standard method and containing the minimum amount of antigen or potency value shall prove its efficacy (protection); each future commercial batch shall be tested before release to ensure it has the same potency value demonstrated by the batch(es) used for the efficacy test(s). Each batch of live vaccine should contain sufficient live mycoplasmas per dose per bird to last until the expiry date.

Vaccine efficacy (protection) should be estimated in vaccinated animals directly by evaluating their resistance to challenge.

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* *

NB: There are WOA Reference Laboratories for avian mycoplasmosis (*Mycoplasma gallisepticum* and *M. synoviae*) (please consult the WOA Web site:

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

Please contact the WOA Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for avian mycoplasmosis (*Mycoplasma gallisepticum* and *M. synoviae*)

NB: FIRST ADOPTED IN 1991 AS MYCOPLASMOSIS (*MYCOPLASMA GALLISEPTICUM*).
MOST RECENT UPDATES ADOPTED IN 2021.

Appendix 1

Avian mycoplasmosis (*Mycoplasma gallisepticum*, *M. synoviae*) Intended purpose of test: population freedom from infection

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Detection of the agent</u>							
Conventional PCR ☒ ☒ ☐ Poultry	(tracheal) swab mgc2 gene (MG) 16S rRNA (MS)	MG Asp = 100% Ase = $1 \cdot 10^3$ UCC/ml MS Asp = 100% Ase = $2 \cdot 10^2$ UCC/ml	Analytical accuracy only was calculated	Carried out by the Reference Laboratories	1. High specificity and sensitivity 2. Rapid 3. Allows detection of non-viable microorganisms 4. Possibility to test sample pools consisting of 10 swabs	1. Requires an electrophoresis gel 2. Requires an operator to acquire results 3. Potential risk of contamination when loading samples on the gel	Lauerman (1998) García <i>et al.</i> (2005) Matucci <i>et al.</i> (2023)
Real-time PCR ☒ ☒ ☒ Poultry	(tracheal) swab mgc2 gene (MG) 16S–23S ISR (MS)	MG Asp = 100% Ase = $6 \cdot 10^3$ UCC/ml MS Asp = 100% Ase = $8 \cdot 10^4$ UCC/ml	Analytical accuracy only was calculated	Carried out by the Reference Laboratories	1. High specificity and sensitivity 2. More rapid than conventional PCR 3. Automatable 4. Reduced risk of contamination due to minimal sample handling 5. Allows detection of non-viable microorganisms 6. Possibility to test sample pools consisting of 10 swabs 7. Automated, standardised result interpretation	1. Requires an equipped laboratory and experienced laboratory personnel	Raviv & Kleven (2009)
Isolation on culture media ☒ ☐ ☐ Poultry	Swab	Asp = 100% Ase – see PCR-DGGE	15 samples tested during the proficiency testing	Test accuracy is calculated after participation in international proficiency testing	1. Provides living strains for further testing 2. Allows the detection of different species	1. Lower sensitivity compared with molecular methods 2. Not able to detect non-viable cells or cells that died during sample transfer to the laboratory 3. Time consuming and labour intensive 4. Requires specific culture media and experienced laboratory personnel 5. Reduced possibility to test sample pools made up of many swabs 6. Possible false negative result if antimicrobials effective against mycoplasmas are administered to the birds	Frey <i>et al.</i> (1968) Catania <i>et al.</i> (2024)

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
PCR-DGGE ☒ ☐ ☐ <u>Poultry</u>	<u>Positive culture broth</u> <u>V3 region of the 16s rRNA gene</u>	<u>Asp = 100%</u> <u>Ase = not applicable</u>	<u>Reference strains</u>	<u>Carried out by the Reference Laboratory in Italy</u>	1. <u>High specificity on cultured bacterial strains</u> 2. <u>Enables the untargeted detection of multiple species in the same sample</u>	1. <u>Time consuming and labour intensive</u> 2. <u>Requires positive mycoplasma culture broths</u> 3. <u>Requires an equipped laboratory and experienced lab personnel</u>	<u>McAuliffe <i>et al.</i> (2005)</u> <u>Catania <i>et al.</i> (2014; 2016)</u>
<u>Detection of immune response</u>							
<u>HI</u> ☒ ☒ ☐ <u>Chicken, Turkey</u>	<u>serum</u>	<u>Varies depending on the antigen used</u>	<u>Not applicable</u>	<u>Not applicable</u>	1. <u>Rapid</u> 2. <u>Economical</u>	1. <u>Requires antigen</u> 2. <u>Requires quantification of haemagglutinin units</u> 3. <u>Requires trained lab personnel</u>	<u>Allan & Gough (1974)</u>
<u>RSA</u> ☒ ☐ ☐ <u>Chicken, Turkey</u>	<u>serum</u>	<u>Varies depending on the antigen used</u>	<u>Not applicable</u>	<u>Not applicable</u>	1. <u>High sensitivity</u> 2. <u>Economical</u> 3. <u>Rapid</u> 4. <u>Easy to perform</u>	1. <u>Has variable specificity, cross reaction may occur.</u>	<u>Allan & Gough (1974)</u>
<u>ELISA</u> ☒ ☒ ☐ <u>Animal species depends on the kit</u>	<u>serum</u>	<u>Varies depending on the commercial kit used</u>	<u>See commercial kit details</u>	<u>Carried out by kit producer</u>	1. <u>Commercially available</u> 2. <u>Rapid</u> 3. <u>Allow the analysis of numerous samples due to their potential for automation</u> 4. <u>Economical</u> 5. <u>High sensitivity and specificity</u> 6. <u>Exhibits high test repeatability</u>	1. <u>Requires a microplate absorbance reader</u>	<u>n.a.</u>

Asp= Analytical specificity; Ase= Analytical sensitivity. *After cultivation, the identification step is performed through PCR-DGGE. Therefore, Asp and Ase of the two methods are bound together.

Appendix 2

Avian mycoplasmosis (*Mycoplasma gallisepticum*, *M. synoviae*)

Intended purpose of test: individual animal freedom from infection prior to movement*

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Detection of the agent</u>							
<u>Conventional PCR</u> ☒ ☒ ☐ <u>Poultry</u>	<u>(tracheal) swab</u> <u>mgc2 gene (MG)</u> <u>16S rRNA (MS)</u>	<u>MG Asp = 100%</u> <u>Ase = $1 \cdot 10^3$ UCC/ml</u> <u>MS Asp = 100%</u> <u>Ase = $2 \cdot 10^2$ UCC/ml</u>	<u>Analytical accuracy only was calculated</u>	<u>Carried out by the Reference Laboratories</u>	<u>1. High specificity and sensitivity</u> <u>2. Rapid</u> <u>3. Allows detection of non-viable microorganisms</u>	<u>1. Requires an electrophoresis gel</u> <u>2. Requires an operator to acquire results</u> <u>3. Potential risk of contamination when loading samples on the gel</u>	<u>Lauerman (1998)</u> <u>García <i>et al.</i> (2005)</u> <u>Matucci <i>et al.</i> (2023)</u>
<u>Real-time PCR</u> ☒ ☒ ☒ <u>Poultry</u>	<u>(tracheal) swab</u> <u>mgc2 gene (MG)</u> <u>16S–23S ISR (MS)</u>	<u>MG Asp = 100%</u> <u>Ase = $6 \cdot 10^3$ UCC/ml</u> <u>MS Asp = 100%</u> <u>Ase = $8 \cdot 10^4$ UCC/ml</u>	<u>Analytical accuracy only was calculated</u>	<u>Carried out by the Reference Laboratories</u>	<u>1. High specificity and sensitivity</u> <u>2. More rapid than conventional PCR</u> <u>3. Automatable</u> <u>4. Reduced risk of contamination due to minimal sample handling</u> <u>5. Allows detection of non-viable microorganisms</u> <u>6. Automated, standardised result interpretation</u>	<u>1. Requires an equipped laboratory and experienced laboratory personnel</u>	<u>Raviv & Kleven (2009)</u>

Asp= Analytical specificity; Ase= Analytical sensitivity.

*Individual diagnosis to declare the absence of infection prior to animal movement is scarcely applicable in the industrial poultry context. Therefore, the table is completed with details of tests that offer higher sensitivity. Not applicable to 1-day-old chicks.

Appendix 3

Avian mycoplasmosis (*Mycoplasma gallisepticum*, *M. synoviae*) **Intended purpose of test: contribute to eradication policies**

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Detection of the agent</u>							
Conventional PCR ☒ ☒ ☐ Poultry	(tracheal) swab mgc2 gene (MG) 16S rRNA (MS)	MG Asp = 100% Ase = $1 \cdot 10^3$ UCC/ml MS Asp = 100% Ase = $2 \cdot 10^2$ UCC/ml	Analytical accuracy only was calculated	Carried out by the Reference Laboratories	1. High specificity and sensitivity 2. Rapid 3. Allows detection of non-viable microorganisms 4. Possibility to test sample pools consisting of 10 swabs	1. Requires an electrophoresis gel 2. Requires an operator to acquire results 3. Potential risk of contamination when loading samples on the gel	Lauerman (1998) García <i>et al.</i> (2005) Matucci <i>et al.</i> (2023)
Real-time PCR ☒ ☒ ☒ Poultry	(tracheal) swab mgc2 gene (MG) 16S–23S ISR (MS)	MG Asp = 100% Ase = $6 \cdot 10^3$ UCC/ml MS Asp = 100% Ase = $8 \cdot 10^4$ UCC/ml	Analytical accuracy only was calculated	Carried out by the Reference Laboratories	1. High specificity and sensitivity 2. More rapid than conventional PCR 3. Automatable 4. Reduced risk of contamination due to minimal sample handling 5. Allows detection of non-viable microorganisms 6. Possibility to test sample pools consisting of 10 swabs 7. Automated, standardised result interpretation	1. Requires an equipped laboratory and experienced laboratory personnel	Raviv & Kleven (2009)
Isolation on culture media ☒ ☐ ☐ Poultry	Swab Living cells	Asp = 100% Ase – see PCR-DGGE	15 samples tested during the proficiency testing	Test accuracy is calculated after participation in international proficiency testing	1. Provides living strains for further testing	1. Lower sensitivity compared with molecular methods 2. Not able to detect non-viable cells or cells that died during sample transfer to the laboratory 3. Time consuming and labour intensive 4. Requires specific culture media and experienced laboratory personnel 5. Reduced possibility to test sample pools made up of many swabs 6. Possible false negative result if antimicrobials effective against mycoplasmas are administered to the birds	Frey <i>et al.</i> (1968) Catania <i>et al.</i> (2024)

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Detection of the agent</u>							
PCR-DGGE ☒ ☐ ☐ <u>Poultry</u>	<u>Positive culture broth</u> <u>V3 region of the 16s rRNA gene</u>	<u>Asp = 100%</u> <u>Ase = not applicable</u>	<u>Reference strains</u>	<u>Carried out by the Reference Laboratory in Italy</u>	<u>See Isolation on culture media above</u>	<u>1. Time consuming and labour intensive</u>	<u>McAuliffe <i>et al.</i> (2005)</u> <u>Catania <i>et al.</i> (2014; 2016)</u>
<u>Detection of immune response</u>							
<u>HI</u> ☒ ☐ ☐ <u>Chicken, Turkey</u>	<u>serum</u>	<u>Varies depending on the antigen used</u>	<u>Not applicable</u>	<u>Not applicable</u>	<u>1. Rapid</u> <u>2. Economical</u>	<u>1. Requires antigen</u> <u>2. Requires quantification of haemagglutinin units</u> <u>3. Requires trained lab personnel</u>	<u>Allan & Gough (1974)</u>
<u>RSA</u> ☒ ☐ ☐ <u>Chicken, Turkey</u>	<u>serum</u>	<u>Varies depending on the antigen used</u>	<u>Not applicable</u>	<u>Not applicable</u>	<u>1. Rapid</u> <u>2. Economical</u>	<u>1. Has variable specificity, cross reaction may occur.</u>	<u>Allan & Gough (1974)</u>
<u>ELISA</u> ☒ ☒ ☐ <u>Chicken, Turkey</u>	<u>serum</u>	<u>Varies depending on the commercial kit used</u>	<u>See commercial kit details</u>	<u>Carried out by kit producer</u>	<u>1. Commercially available</u> <u>2. Rapid</u> <u>3. Economical</u>	<u>1. Requires a microplate absorbance reader</u>	<u>n.a.</u>

Asp= Analytical specificity; Ase= Analytical sensitivity.

Appendix 4

Avian mycoplasmosis (*Mycoplasma gallisepticum*, *M. synoviae*) **Intended purpose of test: confirmation of clinical cases**

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Detection of the agent</u>							
Conventional PCR ☒ ☒ ☒ Poultry	(tracheal) swab mgc2 gene (MG) 16S rRNA (MS)	MG Asp = 100% Ase = $1 \cdot 10^3$ UCC/ml MS Asp = 100% Ase = $2 \cdot 10^2$ UCC/ml	Analytical accuracy only was calculated	Carried out by the Reference Laboratories	1. High specificity and sensitivity 2. Rapid 3. Allows detection of non-viable microorganisms 4. Possibility to test sample pools consisting of 10 swabs	1. Requires an electrophoresis gel 2. Requires an operator to acquire results 3. Potential risk of contamination when loading samples on the gel	Lauerman (1998) García <i>et al.</i> (2005) Matucci <i>et al.</i> (2023)
Real-time PCR ☒ ☒ ☒ Poultry	(tracheal) swab mgc2 gene (MG) 16S–23S ISR (MS)	MG Asp = 100% Ase = $6 \cdot 10^3$ UCC/ml MS Asp = 100% Ase = $8 \cdot 10^4$ UCC/ml	Analytical accuracy only was calculated	Carried out by the Reference Laboratories	1. High specificity and sensitivity 2. More rapid than conventional PCR 3. Automatable 4. Reduced risk of contamination due to minimal sample handling 5. Allows detection of non-viable microorganisms 6. Automated, standardised result interpretation	1. Requires an equipped laboratory and experienced laboratory personnel	Raviv & Kleven (2009)
Isolation on culture media ☒ ☒ ☐ Poultry	Swab Living cells	Asp = 100% Ase – see PCR-DGGE	15 samples tested during the proficiency testing	Test accuracy is calculated after participation in international proficiency testing	1. Allows the detection of different species 2. Provides living strains for further testing	1. Lower sensitivity compared with molecular methods 2. Not able to detect non-viable cells or cells that died during sample transfer to the laboratory 3. Time consuming and labour intensive 4. Requires specific culture media and experienced laboratory personnel 5. Reduced possibility to test sample pools made up of many swabs 6. Possible false negative result if antimicrobials effective against mycoplasmas are administered to the birds	Frey <i>et al.</i> (1968) Catania <i>et al.</i> (2024)
PCR-DGGE ☒ ☐ ☐ Poultry	Positive culture broth	Asp = 100% Ase = not applicable	Reference strains	Carried out by the Reference	1. High specificity on cultured bacterial strains 2. Enables the untargeted detection	1. Time consuming and labour intensive	McAuliffe <i>et al.</i> (2005)

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Detection of the agent</u>							
	<u>V3 region of the 16s rRNA gene</u>			<u>Laboratory in Italy</u>	<u>of multiple species in the same sample</u>		<u>Catania et al. (2014; 2016)</u>
<u>Detection of immune response</u>							
<u>HI</u> ☒ ☐ ☐ <u>Chicken, Turkey</u>	<u>serum</u>	<u>Varies depending on the antigen used</u>	<u>Not applicable</u>	<u>Not applicable</u>	<u>1. Economical</u>	<u>1. Requires quantification of haemagglutinin units</u> <u>2. The need to test two serum samples increases the number of entries into the barn, thereby raising the risk of introducing animal pathogens</u>	<u>Allan & Gough (1974)</u>
<u>RSA</u> ☒ ☐ ☐ <u>Chicken, Turkey</u>	<u>serum</u>	<u>Varies depending on the antigen used</u>	<u>Not applicable</u>	<u>Not applicable</u>	<u>1. Economical</u>	<u>1. The need to test two serum samples increases the number of entries into the barn, thereby raising the risk of introducing animal pathogens</u>	<u>Allan & Gough (1974)</u>
<u>ELISA</u> ☒ ☒ ☐ <u>Chicken, Turkey</u>	<u>serum</u>	<u>Varies depending on the commercial kit used</u>	<u>See commercial kit details</u>	<u>Carried out by kit producer</u>	<u>1. Commercially available</u> <u>2. Economical</u>	<u>1. The need to test two serum samples increases the number of entries into the barn, thereby raising the risk of introducing animal pathogens</u> <u>2. Requires a microplate absorbance reader</u>	<u>n.a.</u>

Asp= Analytical specificity; Ase= Analytical sensitivity.

Appendix 5

Avian mycoplasmosis (*Mycoplasma gallisepticum*, *M. synoviae*) **Intended purpose of test: prevalence of infection – surveillance**

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Detection of the agent</u>							
Conventional PCR ☒ ☒ ☐ Poultry	(tracheal) swab mgc2 gene (MG) 16S rRNA (MS)	MG Asp = 100% Ase = $1 \cdot 10^3$ UCC/ml MS Asp = 100% Ase = $2 \cdot 10^2$ UCC/ml	Analytical accuracy only was calculated	Carried out by the Reference Laboratories	1. High specificity and sensitivity 2. Rapid 3. Allows detection of non-viable microorganisms 4. Possibility to test sample pools consisting of 10 swabs	1. Requires an electrophoresis gel 2. Requires an operator to acquire results 3. Potential risk of contamination when loading samples on the gel 4. More expensive than serological methods	Lauerman (1998) García <i>et al.</i> (2005) Matucci <i>et al.</i> (2023)
Real-time PCR ☒ ☒ ☒ Poultry	(tracheal) swab mgc2 gene (MG) 16S–23S ISR (MS)	MG Asp = 100% Ase = $6 \cdot 10^3$ UCC/ml MS Asp = 100% Ase = $8 \cdot 10^4$ UCC/ml	Analytical accuracy only was calculated	Carried out by the Reference Laboratories	1. High specificity and sensitivity 2. More rapid than conventional PCR 3. Automatable 4. Reduced risk of contamination due to minimal sample handling 5. Allows detection of non-viable microorganisms 6. Possibility to test sample pools consisting of 10 swabs 7. Automated, standardised result interpretation	1. Requires an equipped laboratory and experienced laboratory personnel 2. More expensive than serological methods	Raviv & Kleven (2009)
<u>Detection of immune response</u>							
HI ☒ ☒ ☐ Chicken, Turkey	serum	Varies depending on the antigen used	Not applicable	Not applicable	1. Rapid 2. Economical	1. Requires quantification of haemagglutinin units	Allan & Gough (1974)
RSA ☒ ☐ ☐ Chicken, Turkey	serum	Varies depending on the antigen used	Not applicable	Not applicable	1. Rapid 2. Economical		Allan & Gough (1974)
ELISA ☒ ☒ ☒ Chicken, Turkey	serum	Varies depending on the commercial kit used	See commercial kit details	Carried out by kit producer	1. Commercially available 2. Rapid 3. Economical	1. Requires a microplate absorbance reader	n.a.

Asp= Analytical specificity; Ase= Analytical sensitivity.

Appendix 6

Avian mycoplasmosis (*Mycoplasma gallisepticum*, *M. synoviae*)

Intended purpose of test: immune status in individual animals or populations post-vaccination

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Detection of immune response</u>							
HI ☒ ☒ ☐ Chicken, Turkey	serum	<u>Varies depending on the antigen used</u>	<u>Not applicable</u>	<u>Not applicable</u>	1. <u>Rapid</u> 2. <u>Economical</u>	1. <u>Requires quantification of haemagglutinin units</u>	<u>Allan & Gough (1974)</u>
RSA ☒ ☐ ☐ Chicken, Turkey	serum	<u>Varies depending on the antigen used</u>	<u>Not applicable</u>	<u>Not applicable</u>	1. <u>Rapid</u> 2. <u>Economical</u>	1. <u>Lower specificity</u>	<u>Allan & Gough (1974)</u>
ELISA ☒ ☒ ☐ Chicken, Turkey	serum	<u>Varies depending on the commercial kit used</u>	<u>See commercial kit details</u>	<u>Carried out by kit producer</u>	1. <u>Commercially available</u> 2. <u>Rapid</u> 3. <u>Economical</u>	1. <u>Requires a microplate absorbance reader</u>	<u>n.a.</u>

MEETING OF THE BIOLOGICAL STANDARDS COMMISSION

Paris, 8–12 September 2025

CHAPTER 3.3.7.

FOWL TYPHOID AND PULLORUM DISEASE

SUMMARY

Description and importance of the disease: Pullorum disease of chickens is a bacterial infection caused by *Salmonella enterica* subspecies *enterica* serovar *Gallinarum* biovar *Pullorum* (*Salmonella* Pullorum)¹. These are two distinct biovars, share the same antigenic formula (1,9,12:-) and differ in biochemical reaction patterns. ~~At this time~~ The serovar is referred to as *Gallinarum* or *Pullorum* ~~in some parts of the world and Pullorum in others;~~. In this chapter the serovar will be referred to as *Gallinarum* or *Pullorum* according to the biovar under discussion as this is more meaningful from a clinical and epidemiological perspective, and in agreement with the current version White-Kauffmann-Le Minor scheme. They are host-adapted avian pathogens.

In its acute form, Pullorum disease is almost exclusively a septicaemic disease of young chickens, with high mortality rates. However, the organism may also be associated with disease in turkey poults and may ~~be carried~~ persist subclinically in older birds, ~~or leading~~ to reduced egg production, poor and hatchability ~~and, plus a range of atypical signs in older birds.~~ Ovarian transmission is a major route by which the organism can spread. Game birds, and 'backyard' poultry flocks and wild birds ~~may~~ act as key reservoirs of infection, ~~and~~ Wild birds may also act as vectors for the organism and as such are important in the epidemiology of the disease.

Fowl typhoid in chickens and turkeys is caused by *S. Gallinarum* biovar *Gallinarum* and is more often observed in the later growing period and in mature stock. Disease is often characterised by rapid spread with high morbidity and acute or subacute mortality. Red mites may be involved in the transmission of disease and persistence in poultry houses.

Clinical signs in chicks and poults include anorexia, diarrhoea, dehydration, weakness and death. In mature birds, Pullorum disease is less severe but decreased egg production, poor hatchability and some increased mortality may occur. Fowl typhoid is a more acute septicaemic condition which mainly affects mature birds and may be particularly severe in commercial laying flocks.

Identification of the agent: Samples should not be taken from birds ~~or eggs~~ that have recently been treated with antimicrobial drugs. Swabs or aseptically collected samples from infected tissues, or intestinal and cloacal contents should be used for diagnostic testing. Other materials that may be sampled include eggs, embryos, faecal droppings and hatcher debris, especially fluff, dust and broken eggshells and chick box linings. Samples of tissues such as caecal tonsils, liver, gall bladder and spleen from infected birds are preferable to faecal and environmental samples. Tissue samples should be inoculated into non-selective and selective enrichment broths and on selective agar ~~medium-media,~~ such as brilliant green agar, as soon as possible after collection. In case of delay, samples should be stored at 4°C. Typical colonies can be identified by serological and biochemical tests. Molecular approaches such as polymerase chain reaction (PCR) can also be used to detect, identify and differentiate biovars *S.*-*Gallinarum* and *S.*-*Pullorum*. Final serological confirmation of

1 See the note in Chapter 3.10.3 *Salmonellosis* for the principles followed concerning the nomenclature of *Salmonella*.

suspect isolates and biochemical differentiation among Gallinarum and Pullorum can normally only be completed in a Salmonella Reference Typing Laboratory.

Serological tests: These are satisfactory for identifying the presence and estimating the prevalence of infection within a flock. ~~The test used in the field is the rapid whole blood plate agglutination test~~ is commonly used but. ~~This test is unreliable in turkeys and ducks as many uninfected birds may give positive reactions.~~ In the laboratory a serum agglutination test is used, either as a rapid plate test or as a tube test. These can be applied as macro- or microagglutination tests, though the latter may be more likely to give false-positive results with turkey sera. Any positive reactors should be confirmed as being infected by culture at post-mortem examination. Enzyme-linked immunosorbent assays have been reported but no commercial test is available.

The use of vaccines to control *S. Enteritidis* or *S. Gallinarum* infections in chickens may cause problems in the interpretation of serological results.

Requirements for vaccines: Live and inactivated vaccines are available for fowl typhoid in some countries. One of the most commonly used vaccines is a commercial live vaccine (subcutaneous injection) derived from the stable rough strain of *S. Gallinarum* known as '9R'.

A. INTRODUCTION

Fowl typhoid and pullorum disease, caused by *Salmonella enterica* subspecies *enterica* serovars Gallinarum biovars Gallinarum and Pullorum, respectively, ~~are remain~~ widely distributed throughout the world, especially in developing countries (Barbour *et al.*, 2015; Zhou *et al.*, 2022). These pathogens are increasingly associated with antimicrobial resistance, including multidrug-resistant (MDR) strains resistant to cephadrine, trimethoprim-sulfamethoxazole, and ampicillin where increasing antimicrobial resistance in these strains has also become a problem (EFSA, 2017; Parvej *et al.*, 2016). They have been eradicated from commercial poultry in many developed countries of Western Europe, the United States of America (USA), Canada, Australia and Japan. The move towards free-range production in many countries may increase the risk of infection (Violitz, 2016), but many outbreaks involve intensively housed laying hen or breeding flocks. In the USA and the United Kingdom the serovar is referred to as Pullorum ~~for historical consistency~~ (Hitchner, 2004), even though the strains are now considered to be the same serovar ~~that is derived from S. Enteritidis by gene deletion events (Thomson *et al.*, 2008).~~ Despite the high sequence similarity to S. Enteritidis, both biovars exhibit a relatively large number of predicted pseudogenes. Genomic features indicate a recent evolutionary divergence, possibly originating from a non-motile common ancestor that branched off from the broad-host-range S. Enteritidis lineage, accompanied by adaptations promoting host specificity and systemic, invasive infection (Wales & Lawes, 2023). In this chapter the terms serovar Gallinarum or Pullorum will be used, as this more usefully distinguishes the two biovars that cause clearly distinct clinical syndromes and are therefore epidemiologically different. *Salmonella* Gallinarum outbreaks occurred in some European countries in the first decade of the 21st century (Ivanics *et al.*, 2008). *Salmonella* Pullorum remains as a constant reservoir in wild and game birds (Barrow *et al.*, 2012; Shivaprasad *et al.*, 2013) and outbreaks in commercially reared poultry have been reported in some European countries (Molenaar *et al.*, 2023).

Salmonellosis caused by *Salmonella bongori* or subspecies of *Salmonella enterica* is covered in Chapter 3.10.3 *Salmonellosis*.

The principal host species is chickens (*Gallus gallus*) even though clinical infections also occur in turkeys and pheasants, and outbreaks or sporadic cases are rarely documented in other game birds and some wild species (EFSA, 2017).

Clinical signs of fowl typhoid are typical of a septicaemic condition in poultry and include increased mortality and poor quality in chicks hatched from infected eggs. Older birds show signs of anaemia, depression, laboured breathing and diarrhoea causing adherence of faeces to the vent. The highest mortality in pullorum disease occurs in birds of 2–3 weeks of age. In older birds disease may be mild or inapparent. Signs such as declining feed consumption, droopy appearance, ruffled feathers, pale and shrunken combs may be seen. In breeding and laying flocks susceptibility is increased at the point of lay (Wigley *et al.*, 2005), but reduced egg production, fertility and hatchability may be the only signs of pullorum disease. Trans-ovarian infection resulting in infection of the egg and hatched chicks or poults is one of the most important transmission routes for both diseases (Haider *et al.*, 2014).

Post-mortem signs of pullorum disease in newly hatched chicks are those of peritonitis with generalised congestion of tissues and an inflamed unabsorbed yolk sac. Longer standing infections commonly lead to typhlitis with development of necrotic caecal casts and small necrotic foci in the liver, lungs and other viscera. Small lesions in the liver and spleen of Pullorum-infected birds may show a 'white spot' appearance that is not seen with Gallinarum; however, this lesion is not pathognomic. These *Salmonella* are very relatively poor at colonisation and survival detection in the gastrointestinal tract is often indicative of later stages of clinical disease. Adult birds may develop

misshapen, discoloured and/or shrunken ovaries with follicles attached by pedunculated fibrous stalks. Variant strains of *S. Pullorum* do not normally cause clinical disease or may result in mild, nonspecific signs but may lead to seroconversion.

In fowl typhoid, as well as generalised signs of septicaemia, the liver is usually enlarged, dark and friable with a distinctive coppery bronze sheen that may only develop after exposure to air. The bone marrow is also often dark brown. Although clinical signs and post-mortem findings of pullorum disease and fowl typhoid may be highly suggestive of the conditions, they are not sufficiently distinct from other causes of septicaemia to be pathognomic. It is therefore necessary to confirm disease by isolation or molecular detection (Song *et al.*, 2023) of the organisms. Serological tests can be used to establish the presence of the disease in a flock.

1. Zoonotic risk and biosafety requirements

Salmonella Gallinarum and *S. Pullorum* are host adapted to avian species (Eswarappa *et al.*, 2009) and are considered to pose a minimal zoonotic risk (EFSA, 2017; Shivaprasad, 2000). There have been a few case reports of human illness (showing fever and diarrhoea) due to *S. Pullorum*, typically via infected eggs. Moreover, rarely systemic human infection with *S. Gallinarum* has been documented. No evidence is available of interpersonal transmission of these biovars (EFSA, 2017). Although the genome is adapted to a non-intestinal environment and has lost flagella genes to help evade host immune responses (Lopes *et al.*, 2016), it is they are continually evolving, which could theoretically widen the host range in future (Liu *et al.*, 2002; Campos *et al.*, 2024). Non-typhoidal *Salmonella* serovars should be handled with appropriate biosafety and containment procedures as determined by biological risk 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 fowl typhoid and Pullorum disease 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						
Bacterial isolation	✓✓✓	✓✓✓	✓✓✓	✓✓✓	✓✓✓	□□□
Rapid alternative methods e.g. PCR	✓□□-✓✓✓	✓□□-✓✓✓	✓□□-✓✓✓	✓□□-✓✓✓	✓□□-✓✓✓	□□□
Detection of immune response						
<u>Whole blood agglutination</u> WBT	✓✓□	□□□	✓✓✓	□□□	✓□□	✓✓□
<u>Rapid serum agglutination</u> RSA	✓✓□	□□□	✓✓□	□□□	✓□□	✓✓□
<u>Tube agglutination</u> -SAT	✓✓□	□□□	✓✓□	□□□	✓✓□	✓✓✓
<u>Micro-agglutination</u>	✓✓□	□□□	✓✓□	□□□	✓✓□	✓✓✓
ELISA	✓□□	□□□	✓□□	□□□	✓□□	✓✓□

Key: ✓✓✓ = recommended for this purpose; ✓✓□ = recommended but has limitations;

✓□□ = suitable in very limited circumstances; □□□ = not appropriate for this purpose.

PCR = polymerase chain reaction-based tests; WBT = whole blood agglutination test; RSA = rapid slide agglutination test;

SAT = serum agglutination test; ELISA = enzyme-linked immunosorbent assay.

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

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

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

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

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

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

1. Identification of the agent

1.1. Bacteriological culture methods

In the acute stages of disease, the agent of both diseases can be recovered from almost all organs, tissues and faeces. In older birds that have become carriers, *S. Pullorum* is most commonly recovered from the ova and oviduct; and it is recovered only occasionally from other organs and tissues, including the alimentary tract. In the acute phase of fowl typhoid the organism is also widely distributed, but in carrier birds, the organism is found most often in the liver, spleen and reproductive tract, and occasionally in the caecal tonsils.

Salmonella Pullorum and *S. Gallinarum* belongs to the White-Kauffmann–Le Minor scheme serogroup D, along with *S. Enteritidis*, which is closely related (Grimont & Weill, 2007). The organisms are Gram-negative non-sporogenic rods 1.0–2.5 µm in length and 0.3–1.5 µm in width. They are considered to be non-motile under normal conditions, but inducement of flagellar proteins and motility has been ~~shown~~ reported in some strains of *S. Pullorum* when grown in special media (Holt & Chaubal, 1997). Differentiation between *S. Gallinarum* and *S. Pullorum* is crucial from an epidemiological and preventive standpoint.

For optimal recovery of the organisms, the birds being sampled should not have been treated with antimicrobial drugs for approximately 2–3 weeks previously.

Samples may be obtained from live birds, preferably after identifying them as highly sero-positive birds. Fresh or freshly chilled carcasses, egg materials, fresh faeces, organs such as liver, spleen, yolk sac or any contaminated materials from housing, incubators or transport boxes may also be taken, but faecal and environmental samples often fail to reveal the presence of the organism because of inconsistent shedding and the poor sensitivity of bacteriological detection methods for samples that include a complex faecal flora². Swabs may be taken from the cloaca of sick live birds but post-mortem tissues are preferable. Samples from visibly abnormal tissues are preferred, but aseptically gathered samples can also be taken from the spleen, liver, gall-bladder, kidneys, lungs, heart, ova, testes, alimentary tract or joint lesions. The preferred tissues for routine investigation are liver, ileo-caecal junction and ovaries/oviduct. The surface is seared with a hot spatula and a sample is obtained by inserting a sterile cotton swab or sterile loop through the heat-sterilised surface. The demonstration of infection in serological reactor birds that are apparently normal may, in some cases, require the culture of large volumes of homogenised tissues as well as direct swabbing. Tissue pools may be made from tissues collected from a number of birds, and, for routine testing, five ileo-caecal junction samples may be pooled. Larger numbers of aseptically collected non-intestinal samples can be pooled, but for practical purposes, composite samples of liver, spleen and ovary from five birds are often tested.

When floor litter, faecal material or hatchery material such as hatcher basket liners is sampled, it should be remembered that *S. Pullorum* and *S. Gallinarum* in low numbers associated with subclinically infected carrier birds are more difficult to isolate from faecal and environmental samples than other salmonellae and it is always preferable to culture sick or recently deceased birds or 'dead in shell' embryos. Red mites associated with poultry that are infected with *S. Gallinarum* often contain the organism after feeding and can be cultured. These samples should be cultured by direct inoculation of a selective enrichment broth such as selenite cysteine or selenite F, followed by plating on selective media such as brilliant green agar (Parmar & Davies, 2007; Proux et al., 2002), or polymerase chain reaction (PCR) can be used (Cocciolo et al., 2020; Song et al., 2023) for their detection.

Both *S. Pullorum* and *S. Gallinarum* grow well in pure culture on non-selective media, but selective and enrichment media have been described that contain substances to inhibit the growth of extraneous organisms. *Salmonella Pullorum* may grow slowly and produce very small colonies on selective media so incubation of plates for 48 hours is recommended. The efficiency of recovery of *Salmonella* varies according to circumstances, and experience in the use of a medium is an important but unquantifiable factor. Some complex media may have an inhibitory effect on these organisms, so that it is advisable to use both non-selective and selective media for isolation from tissues. Both solid media and broths can be employed. As the toxic properties of selective media may vary, it is preferable to monitor these by comparing growth of control cultures on both types of medium. The inhibitory media should grow at least

2 A combination of sample types and agent identification methods applied on the same clinical sample is sometimes recommended.

75% of the colonies of the corresponding non-inhibitory medium (~~Ellis et al., 1976;~~ Mallinson & Snoeyenbos, 1989).

All the media mentioned below are examples of commonly used media, but there are many others found to be equally satisfactory and it is recommended that the most suitable products are validated locally in relation to strains that are circulating in a particular region.

Non-inhibitory media include nutrient agar and blood agar, on which colonies are seen to be smooth, translucent, slightly raised, and about 1–2 mm in diameter. *Salmonella* Gallinarum grows more rapidly than *S. Pullorum* and produces larger colonies with a distinctive smell resembling that of seminal fluid on most media. Broths include buffered peptone water and nutrient and meat infusion broths or universal pre-enrichment broth.

1.1.1. Selective media

i) MacConkey agar

MacConkey agar is largely inhibitory to non-enteric organisms; it differentiates lactose fermenters (pink colonies) from non-lactose fermenters (colourless colonies). NaCl is omitted to limit the spread of *Proteus* colonies. *Salmonella* colonies are smooth and colourless. *Salmonella* Pullorum produces smaller colonies than other salmonellae. MacConkey is the agar of choice for direct plating from tissues.

ii) Xylose lysine deoxycholate agar

This agar is inhibitory to non-enteric organisms. *Salmonella* Pullorum grows sparsely as small red translucent colonies. *S. Gallinarum* colonies are small, dome-shaped, and may have a central black spot due to H₂S production, but this reaction may be delayed or variable.

iii) Brilliant green agar

Brilliant green agar (BGA) is inhibitory to coliforms and most *Proteus* strains; useful for distinguishing enteric organism colonies. Salmonellae form low, convex, pale red, translucent colonies of 1–3 mm in diameter, similar to *Citrobacter*. *Proteus* forms pin-point colonies, *Pseudomonas aeruginosa* appears as small red colonies, and lactose fermenters are green. *Salmonella* Pullorum produces smaller more pale colonies than other salmonellae. BGA is the agar of choice following enrichment, but low numbers of *Salmonella* can be easily overgrown by competing organisms when BGA is used for faecal and environmental samples.

iv) Brilliant green sulphydryl agar

This agar is inhibitory to coliforms and *Proteus* strains. The sulphydryl is added to stabilise selectivity in the presence of egg materials. *Salmonella* Pullorum produces small colonies.

Salmonella Pullorum and Gallinarum grow poorly and do not produce typical colonies on some ~~newer~~ earlier chromogenic agars such as Rambach agar (Wales & Lawes, 2023), but more recently developed chromogenic media with a wider detection range could be evaluated for suitability.

1.1.2. Liquid enrichment and selective media

i) Selenite cysteine and F broths

These broths are inhibitory to coliforms but not *Proteus* and are improved by addition of brilliant green. Loss of activity after 24 hours limits its use. Selenite cysteine broth is more stable and less inhibitory than selenite F broth, so is normally preferable (e.g. for hatchery-based monitoring of hatcher basket liners or meconium) except in the case of fresh faecal samples from mature birds where highly competitive flora may be present. Although selenite-based broths are considered to be preferable for isolation of *S. Pullorum* and *S. Gallinarum* from faeces by direct enrichment (Shivaprasad *et al.*, 2013), if there are difficulties with issues of toxicity or shelf life in particular laboratories the other enrichment broths mentioned below may be used. Most of these other broths are however designed to be used following a non-selective enrichment stage and *S. Gallinarum* and *S. Pullorum* are readily overgrown by competitor organisms in non-selective faecal culture resulting in false-negative tests. Direct selective enrichment is therefore recommended for faeces and intestinal or environmental samples. Non-selective enrichment may give better results for

tissues obtained by aseptic post-mortem where there should be no competing organisms (Mallinson & Snoeyenbos, 1989). ISO 6579-1:2017 – Annex D suggests using selenite cysteine medium in addition to Rappaport–Vassiliadis soya (RVS) broth and Muller-Kauffmann Tetrathionate-Novobiocin Broth (MkTTn) broth for the detection of *S. Gallinarum* and *S. Pullorum* biovars. Sodium hydrogen selenite is potentially teratogenic and may harm the unborn child. Hence, appropriate protective precautions should be taken when preparing and handling this medium.

ii) Tetrathionate/brilliant green broth

This broth is inhibitory to coliforms and *Proteus*, but may also inhibit some strains of *S. Pullorum*/*Gallinarum*.

iii) Rappaport–Vassiliadis soya (RVS) peptone broth

This broth is normally only used for selective enrichment following pre-enrichment but is more stable than selenite broth; use 1 part inoculum to 100 parts medium. *Salmonella* *Pullorum* and *Gallinarum* are more likely to be overgrown by other organisms during pre-enrichment of faeces or intestinal contents than salmonellae that are not host-adapted, so direct enrichment with RVS may also be attempted.

1.2. Recovery of Salmonellae

The methods for recovering *S. Pullorum* and *S. Gallinarum* vary according to the origin of the samples. Although their isolation from cloacal swabs and faeces may be unrewarding, examination of tissues taken at post-mortem is usually more successful (Wales & Lawes, 2023). ~~The methods are described.~~

1.2.1. Cloacal swabs and fresh faeces from live birds

Swabs dipped in nutrient broth are suitable; small swabs being used for young chickens. The swabs are streaked on selective media, and placed in enrichment broth. The plates and the broth are incubated at 37°C. Higher temperatures may be used with some broths, e.g. 41.5°C for RVS. Subcultures are made on to selective media after 24 and 48 hours.

1.2.2. Gall-bladder contents

Swabs of gall-bladder contents are streaked on to non-selective and selective agars and placed in inhibitory and non-inhibitory broths, followed by incubation at 37°C and subculture on to selective agar after 24 and 48 hours.

1.2.3. Organs and tissues

Swabs or segments of tissues are taken in an aseptic manner from individual tissues and lesions and cultured on to non-selective and selective media, and into similar non-selective and selective broths. For suspected infected breeding or rearing flocks, selection of birds for post-mortem examination is usually guided by serological testing. These Samples are incubated at 37°C and subcultured on to selective agar after 24 and 48 hours. Parallel incubation at a higher temperature, e.g. 40–42°C, can also be used to enhance the overall isolation rate. Carrier birds: larger amounts of material may be required to identify the carrier birds. The ovary and oviduct are the tissues of choice for *S. Pullorum*, and the liver, gall-bladder and caecal tonsils as well as ovary and oviduct should be tested for *S. Gallinarum*. In practice it is usually best to pool samples from a variety of tissues including the spleen, but intestinal tissues should not be pooled with other tissues. Tissues are homogenised in a small volume of broth and directly plated out. Approximately 10 ml of homogenate is also added to 100 ml of non-selective enrichment broth (e.g. buffered peptone water) and selective enrichment broth (e.g. selenite cysteine broth or brilliant green broth), and incubated at 37°C. These broths are subcultured on to non-selective and selective agar after 24 hours.

1.2.4. Alimentary canal, including the caecal tonsils and intestinal contents

After grinding or homogenisation in a small volume of broth, 10 ml of the homogenate is incubated in 100 ml of selective enrichment broth at 37°C. In general, better isolation is achieved with selenite cysteine broth.

1.2.5. Eggshells

The broken eggshells are placed in a tenfold volume of enrichment broth (e.g. selenite cysteine broth). The broth is incubated at 37°C and subcultured on to selective agar after 24 and 48 hours.

1.2.6. Egg contents

Aseptically taken contents of fresh eggs are homogenised and mixed with 200 ml of buffered peptone water or nutrient broth, incubated at 37°C, and subcultured on to non-selective and selective agar after 24 and 48 hours. Incubated eggs, whether infertile or containing small embryos, can be similarly treated.

1.2.7. Embryos

Homogenised viscera and swabs from the yolk sacs of well-developed embryos may be streaked on to non-selective and selective agar, one swab being placed in 10 ml of both non-selective and enrichment broth (e.g. selenite cysteine broth or brilliant green broth). Incubation is carried out at 37°C, and subcultures are made on to non-selective and selective agars after 24 and 48 hours.

1.2.8. Environmental samples

These include hatcher fluff, debris and macerated egg/chick waste samples and chick box liners or floor faecal or litter samples; 25 g is mixed with 225 ml of enrichment broth (e.g. selenite cysteine broth, brilliant green broth), incubated at 37°C, and subcultured on to selective agar after 24 and 48 hours.

1.3. Confirmatory procedures

Typical *S. Gallinarum* colonies on non-selective media are round, translucent, glistening, domed, smooth, and 1–2 mm in diameter after 24–48 hours' incubation. *Salmonella* Pullorum colonies are slightly smaller and translucent. On selective media their appearance varies with the medium, but suspect colonies can be investigated serologically by testing for 'O'9 somatic antigens, observing for motility and testing biochemically.

After incubation for 20–24 hours, the plates should be examined carefully for the presence of typical *S. Pullorum* and *S. Gallinarum* colonies. The plates should be re-incubated for a further 24 hours and examined again. For biochemical and serological confirmation from each plate, five typical or suspect colonies should be chosen for further examination. If there are fewer than five typical or suspect colonies, all of them should be tested. Selected colonies should be inoculated onto the surface of nutrient agar, in a manner that allows the growth of separate colonies. For biochemical confirmation, only pure cultures taken from non-selective media should be used. The following media should be inoculated using an inoculating loop: triple sugar iron (TSI) agar; lysine iron agar (or L-lysine decarboxylation medium); urea agar according to Christensen; tryptone/tryptophan medium for indole reaction; glucose with an inverted Durham tube for acid and gas production; dulcitol, maltose, ornithine decarboxylation medium and semisolid agar, for motility. The reactions shown in Table 2 occur.

Identification kits are commercially available for *Enterobacteriaceae*. ~~Molecular tests using ribotyping techniques and PCR have been developed in research laboratories (Kang et al., 2012), and can be used for confirmation and differentiation between *S. Gallinarum* and *S. Pullorum*.~~

For serological confirmation to serogroup level, colonies from non-selective media (nutrient or blood agar) are used. The first stage is elimination of autoagglutinable strains. For this, material taken from a single colony of pure culture is transferred to a glass slide and mixed with a drop of sterile saline. The slide is rocked gently or the drop stirred with a loop for 30–60 seconds and observed for agglutination against a dark background, preferably with the aid of a magnifying glass or dissecting microscope. If the bacteria have clumped into more or less distinctive units, the strain is considered to be autoagglutinable and must not be submitted for the following tests. If the bacterial sample is recognised as non-autoagglutinable, it is tested with a polyvalent 'O' (A–G) antiserum. For this purpose, the material from a single colony is dispersed in the drop of polyvalent 'O' antiserum on the glass slide to obtain a homogenous and turbid suspension. After gently rocking for 30–60 seconds, the reaction is observed against a dark background for agglutination. Alternatively the slide agglutination test may be carried out with smaller volumes of suspension under a dissecting microscope. In this case a portion of the colony to be checked is added to a loopful of saline on the microscope slide to produce a light suspension to check for autoagglutination ('rough strains'). If no agglutination takes place, one or two loops of antisera are added, the drop stirred with a loop and observed for agglutination. *Salmonella* Pullorum and *S. Gallinarum* should agglutinate with polyvalent 'O' antisera but not with polyvalent flagella (poly 'H' phase 1 and phase 2) antisera. If the reaction is positive, the single colony is tested further in the same

manner using group-specific sera for *S. Pullorum* and *S. Gallinarum* serovar ('O'9 antiserum). After serogrouping, isolates may be sent to a reference laboratory for serotyping. The approach described in the ISO TR 6579-3: 2014 'Guidelines for serotyping of *Salmonella* spp.' can be followed for the identification of the antigenic formula.

PCR-based tests may also be used to confirm the serovar, differentiate among *Gallinarum* and *Pullorum* biovars, or the vaccinal status, but have not been fully validated internationally (Batista *et al.*, 2016; Coccio *et al.*, 2020; Kang *et al.*, 2012; Song *et al.*, 2023; Xiong *et al.*, 2016).

Table 2. Biochemical investigation of *Salmonella* Pullorum and *S. Gallinarum*

	<i>Salmonella</i> Pullorum	<i>Salmonella</i> Gallinarum
TSI glucose (acid formation)	+	+
TSI glucose (gas formation)	V	–
TSI lactose	–	–
TSI saccharose	–	–
TSI hydrogen sulphide	V	v
Gas from glucose (medium with Durham tube)	+	–
Urea hydrolysis	–	–
Lysine decarboxylation	+	+
Ornithine decarboxylation	+	–
Maltose fermentation	– or late +	+
Dulcitol	–	+
Motility	–	–

+ = 90% or more positive reaction within 1 or 2 days; – = No reaction (90% or more); v = Variable reactions.

~~It is also possible to confirm and differentiate *S. Gallinarum* by specific PCR (Kang *et al.*, 2012).~~

1.4. Test procedure for culture of visceral, faecal, intestinal and environmental samples for *S. Pullorum* and *S. Gallinarum*

- i) Where possible, begin laboratory procedures on the same day as samples are collected.
- ii) Homogenise the material as much as possible by manual mixing, gentle macerating or stomaching with a small volume of sterile saline if the material is dry.
- iii) Stir the mixture with a small rectal swab or loop and streak thickly on to one-quarter of a brilliant green agar plate. (Swabs from uncontaminated tissues sampled in an aseptic manner can also be streaked on to blood agar).
- iv) From this deposit of material on the plate, streak the rest of the plate to obtain individual colonies.
- v) Add 5–25 g of the homogenised sample to freshly made selenite cysteine broth (see note on liquid enrichment and selective media above) to make a 1:10 sample to broth ratio. Shake or stir to disperse the sample in the broth.
- vi) Incubate the brilliant green agar plates and selenite cysteine broth at 37°C for 24 hours.
- vii) Examine the plate after 24 hours' culture.
- viii) Carry out agglutination tests on up to five suspect colonies with polyvalent 'O' (A–G) antisera and polyvalent H (phase 1 and phase 2) antisera. If agglutination is unclear subculture suspect colonies on to nutrient agar or blood agar and repeat tests after 24 hours' incubation of those media. If poly 'O' is positive then check with 'O'9 antiserum. If 'O'9 is positive and poly 'H' is negative, this is indicative of the possible presence of *S. Pullorum* or *S. Gallinarum*.
- ix) If there are no positive colonies on the brilliant green agar plate, streak out a 10 µl loop of incubated selenite cysteine broth onto brilliant green agar as in step iv above.
- xi) Incubate the brilliant green agar plates at 37°C for 24 hours and re-incubate the previous (negative) brilliant green agar plates and the selenite cysteine broths for a further 24 hours.
- xi) Repeat examination of plates as in step vii above.

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- xii) If plates are still negative, re-plate from selenite cysteine broth and incubate brilliant green agar plate, that was inoculated in step ix, for a further 24 hours and examine as in step vii above.
 - xiii) Confirm *S. Pullorum* and *S. Gallinarum* using biochemical tests as shown in Table 2. Isolates can be sent to a *Salmonella* reference laboratory for confirmation of serotype and for further molecular typing if required for epidemiological purposes.

1.5. Molecular epidemiology

Standard molecular 'fingerprinting' techniques used for *Salmonella*, such as plasmid profile analysis, pulsed field gel electrophoresis, PCR-restriction fragment length polymorphism (RFLP) or ribotyping can be used for investigating outbreaks of *S. Pullorum* or *S. Gallinarum*. It is often necessary to use combinations of such methods and different restriction enzyme combinations to obtain maximum discrimination because of a high level of clonality. The most effective techniques may also vary by country because of the nature of circulating clones in that region. High throughput whole genome sequencing has also been applied to *S. Gallinarum*, but is not yet available or economically viable in all countries (~~De Cari et al., 2016~~ Molenaar et al., 2023). If available, this method should be considered the gold standard to understand the molecular epidemiology of *S. Pullorum* or *S. Gallinarum*.

2. Serological tests

Serological tests are best applied as a flock test as results for individual birds will vary according to the stage of infection. It is therefore necessary to take sufficient individual samples to determine infection in the flock. The number of samples will depend on the expected prevalence and level of confidence desired (see Chapter 1.1.2 *Collection, submission and storage of diagnostic specimens* and Chapter 1.1.3 *Transport of biological materials*). If the test is to be used for detecting individual infected birds for culling, it should be repeated at least twice and preferably until the whole flock has given at least two negative tests.

The tests that are most readily applied include rapid whole blood agglutination, rapid serum agglutination, tube agglutination and micro-agglutination (~~USDA, 1996~~). Other invasive *Salmonella* such as *S. Enteritidis* and *S. Typhimurium* or use of vaccination may lead to false-positive results in serological tests for *S. Pullorum*.

Both *S. Pullorum* and *S. Gallinarum* possess 'O' antigens 9 and 12 and may also possess O antigen 1 (~~Brooks et al., 2008~~). However, in the case of *S. Pullorum*, there is a variation in the ratio of 12₁, 12₂ and 12₃; the standard strain contains more 12₃ than 12₂, while the reverse is true of the variant form. Intermediate forms also exist. (There appears to be no such form variation in the case of *S. Gallinarum*.) As this variation occurs, it is necessary to use a polyvalent antigen in immunodiagnostic tests. The same antigen is used to detect both *S. Pullorum* and *S. Gallinarum*, but detection of the latter may be relatively poor (~~Proux et al., 2002~~). Investigation of other target antigens could therefore be beneficial, both for serological detection and vaccine development (Jiang et al., 2023).

2.1. Rapid whole blood agglutination test

The rapid whole blood agglutination test can be used under field conditions for detecting both *S. Pullorum* and *S. Gallinarum*, and the reactors can be identified immediately. However, it is not reliable in turkeys or ducks as the test results in a significant proportion of false-positive results. Sera can be screened by rapid slide agglutination test and positive reactions confirmed by the more specific tube agglutination test. Chickens can be tested at any age, although some authorities specify a minimum age of 4 months (Shivaprasad et al., 2013; ~~USDA, 1996~~) and positive results from chicks less than 4 weeks of age may be due to maternal antibodies.

2.1.1. Preparation of stained antigen for the rapid whole blood or rapid serum agglutination test

Incubate one standard form strain of *S. Pullorum* (antigenic structure 9, 12₁, 12₃) and one variant form (antigenic structure 9, 12₁, 12₂) at 37°C and harvest separately until final mixing for the complete antigen.

Sow strains on to separate agar slopes, incubate at 37°C for 24 hours, emulsify growth with sterile normal saline and spread an inoculum over an agar plate to produce easily selected discrete colonies. For this the plates are incubated for 48 hours, a number of colonies are marked out and each is tested for agglutination on a slide with 1/500 acriflavine in saline. Smooth-phase colonies do not produce agglutination. Pick off typical colonies that do not produce any agglutination, seed on to agar slopes, and incubate for 24 hours. Emulsify the growth in saline and evenly distribute 2 ml over the surface of the medium (200 ml) in a Roux or similar flask. Incubate the flasks for 60 hours.

For harvesting the bacterial growth, flood the surface of each flask with approximately 10 ml sterile buffered formol saline, pH 6.5 (8.5 g/litre sodium chloride, 10 ml/litre neutral formalin, 4 ml/litre

0.5 M sodium phosphate: made up to 1 litre with distilled water, pH adjusted to 6.5 using 1 M orthophosphoric acid or 1 M sodium hydroxide), to give dense cell suspensions. Add 12–15 sterile glass beads of 3–5 mm diameter and rock the flasks until all the culture is in even suspension; leave in a vertical position for at least 15 minutes. Check the morphology and purity of the suspensions by preparing and examining Gram-stained films. Bulk the suspension from each flask containing the same strains. To each 100 ml of suspension, add 200 ml of absolute alcohol. Shake the mixture and allow to stand for 36 hours, or until precipitation is complete. Check the agglutinability of the standard and variant precipitate by first centrifuging a sample to separate the alcohol, which is removed, dilute with normal saline and test with a known positive and negative serum. If satisfactory, remove the clear supernatant alcohol (centrifugation at 2000 *g* for 10 minutes may be helpful for precipitation), and add sufficient phosphate buffered saline (PBS) containing 10% (v/v) glycerol to standardise the density to 75 × No. 1 Wellcome opacity tube (or 50 × tube No. 1.0 on the McFarland scale). Add equal volumes of standard and variant strains, and add 1% (v/v) of 3% (w/v) alcoholic crystal violet solution to the final mixture, and allow to stand for 48 hours at room temperature. Store in a tightly closed container at 0–4°C for up to 6 months. To assess safety, carry out a culture test on blood agar to confirm non-viability of the unwashed antigen before standardisation. Each bottle of antigen must be tested after alcoholic precipitation and before standardisation against standard titre antisera for *S. Pullorum* and *S. Gallinarum*, and against a negative serum. If possible, also test with known positive and negative serum and blood from positive and negative chickens.

Stained antigen products for the whole blood plate agglutination test are available commercially, and although there seem to be some slight differences in their sensitivity ([Cast, 1997](#)), it is unlikely that poultry flocks infected with the different variants of *S. Pullorum* would be missed.

2.1.2. Test procedure

- i) Use a clean white tile marked into squares of about 3 × 3 cm. If a tile with 3 × 4 squares is used, up to 12 blood samples can be tested at the same time.
- ii) Place 1 drop (about 0.02 ml) of crystal-violet-stained antigen in the centre of each square.
- iii) Obtain a sample of fresh whole blood. This is conveniently done by making a stab of a wing vein using a needle with a triangular point.
- iv) Place an equal size drop of fresh whole blood next to a drop of antigen.
- v) Mix the drops of antigen and blood using a fine glass rod, which is wiped clean between samples.
- vi) Use a gentle rocking motion to keep the drops agitated for up to 2 minutes. Several tests may be carried out simultaneously on the same tile, but the drops should not be allowed to dry out during this time. In very warm conditions, a smaller number of larger drops per plate may be required to avoid drying out.
- vii) A positive reaction is indicated by easily visible clumping of the antigen within 2 minutes.
- viii) A negative reaction is indicated by absence of clumping of the antigen within 2 minutes.
- ix) Include known positive and negative control sera on each testing occasion, using them in the same way as the blood.
- x) On completion of a set of tests, the tile is washed and dried, ready for further use.

In the absence of positive reactions, any inconclusive reactions can only be interpreted in the light of the previous *Salmonella* testing history of the flock. Where there are positive reactors, any doubtful reactor should be regarded as positive. Also, recently infected birds may not show a typical positive reaction until they are retested after 3–4 weeks.

2.2. Rapid serum agglutination test

The RST-rapid serum agglutination (e.g. rapid slide agglutination) is performed in the same manner, except that serum is substituted for whole blood. For export test purposes an initial screening of sera by RST-rapid serum agglutination followed by confirmation of positives by the tube agglutination test is the optimal approach. Ideally serum samples tested by any method should be tested within 72 hours of collection as nonspecific reactions may increase in older samples. Fresh samples can be frozen for later testing if a delay is unavoidable.

2.3. Tube agglutination test

Fresh serum from chickens, turkeys or other birds is used at an initial dilution of 1/25, obtained by mixing 0.04 ml of serum with 1.0 ml of antigen³. Positive and negative control sera are included in each test. The antigen is prepared from unstained *S. Pullorum* or *S. Gallinarum* cultures diluted to a concentration of No. 1 on the McFarland scale (as described above). The mixture is incubated at 37 or 50°C for 18–24 hours before reading. A positive reaction consists of a granular white deposit with a clear supernatant fluid; a negative reaction shows uniform turbidity. Samples positive at a dilution of 1/25 are retested at a higher range of dilutions and a titre of 1/50 is usually considered to be positive, although this figure seems to vary in the literature. In many cases a single dilution of 1/50 is used but this may fail to detect some flock infections if only small numbers of samples are taken.

2.4. Micro-agglutination test

This resembles the tube agglutination test, but requires much smaller volumes of reagents. The test is performed in microtest plates. Sera are first diluted by adding 10 µl of serum to 90 µl of normal saline, and then adding 100 µl of previously standardised stained microtest antigen to give a final dilution of 1/20. By titrating the serum in doubling dilutions and adding an equal volume of standardised stained antigen, an end-point (titre) can be obtained. The plates are sealed and incubated at 37°C for 18–24 or 48 hours. A positive reaction consists of a fine diffuse precipitation, whereas a negative reaction shows a button-like precipitate. Titres of 1/40 are usually considered to be positive but this test is more liable to produce false-positive results with turkey sera.

Other serological tests include micro-antiglobulin (Coombs), immunodiffusion, haemagglutination and enzyme-linked immunosorbent assay (ELISA).

ELISA techniques have been described for detecting antibodies to *S. Pullorum* and *S. Gallinarum* (Oliveira *et al.*, 2004). The indirect ELISA using lipopolysaccharide antigen is likely to be the most sensitive and specific serological flock test for *Salmonella*, including *S. Gallinarum* and *S. Pullorum*. It is relatively easy to perform with serum or yolk, and can be used for quantifying the titre of antibody (Barrow, 1992; 1994). No specific commercial ELISA kits for *S. Pullorum* and *S. Gallinarum* are currently available, but an indication of likely infection can often be obtained using a lipopolysaccharide (LPS)-based commercial ELISA for *S. Enteritidis* or *Salmonella Group D*; these tests have not been validated for this purpose.

C. REQUIREMENTS FOR VACCINES

1. Background

1.1. Rationale and intended use of the product

Although both live and inactivated vaccines have been prepared for use against *S. Gallinarum* (Paiva *et al.*, 2009), the vaccine most widely used is made from the ~~rough-9R strain (Harbourne *et al.*, 1963; Revollo & Ferreira, 2012).~~ The lipopolysaccharide (LPS) structure of SG9R has a semi-rough texture that decreases the pathogenicity of the vaccinal strain. It is normally only employed in chickens. The number of viable organisms per dose is important; these organisms can survive in vaccinated birds for many months and may be transmitted through the egg (and perhaps from bird to bird). Vaccination may reduce flock losses, but will not prevent infection with field strains. In addition, vaccination with 9R may sometimes precipitate high mortality in infected birds (Silva *et al.*, 1981), and may stimulate the production of transient antibodies. It is usual to vaccinate at 8 weeks and again at 16 weeks of age. Antimicrobials should be avoided before and after vaccination.

Currently available vaccines, however, have only a minor role to play in the control of fowl typhoid as they offer short-lived protection against clinical disease and limited or variable protection against infection. Autogenous or locally produced vaccines can also be used to control clinical disease, but care must be taken to avoid strain instability leading to reversion to virulence (~~Okamoto *et al.*, 2010; van Immerseel *et al.*, 2013).~~ Control can best be achieved by biosecurity, hygiene, good management, monitoring and removal of infected flocks, although the routine vaccination against *S. Enteritidis* and *S. Typhimurium* that is carried out in breeding and laying hen flocks in many countries may be partially protective against introduction of *S. Gallinarum* (Lee, 2015). Commercially available 9R vaccines have been used for reduction of *S. Enteritidis* in laying flocks in some countries but may be prohibited or are not commercially available in some countries where fowl typhoid is not present (Lee *et al.*, 2005). Even in countries with fowl typhoid, use of vaccine may complicate control as it does not prevent infection,

3 For preparation of small volumes of somatic antigens see chapter 3.10.3.

only reduce clinical disease and allow production to continue from infected flocks. It is therefore preferable to aim for eradication of the organism rather than acceptance of on-going disease, but this is often not economically viable in large multi-age holdings as eradication of red mites is necessary to ensure continued freedom from infection (~~Wales et al., 2010~~).

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.

Most vaccines are produced in highly industrial commercial processes and are regulated by national veterinary medicines licensing authorities. Smaller quantities of emergency ~~herd-flock~~ vaccines or autogenous vaccines are produced by private laboratories, but each production has to be specifically licensed. It is recommended that a validated commercial vaccine is used unless there is no alternative because of the need to maintain quality and avoid risk associated with reversion to virulence. Live vaccines must also be bacteriologically distinguishable from field strains or surveillance and control programmes may be compromised. Observations from some countries suggest that it is not always straightforward to distinguish between *S. Gallinarum* vaccines and field strains (van Immerseel *et al.*, 2013). There will inevitably be some interference with serological monitoring for *S. Gallinarum* and potential interference with serological monitoring for *S. Enteritidis*, unless a stepwise approach is used in which a sensitive LPS-based ELISA is used to test for antibodies to O9 antigens and positive sera are further tested with a flagella antigen ELISA, which will give a negative reaction in cases of *S. Gallinarum* infection (Shivaprasad *et al.*, 2013). ~~Recent Ongoing~~ work on the molecular mechanisms of infection should lead to the development of ~~improved~~ vaccines with improved efficacy and safety in future (~~Barrow et al., 2012 Pan et al., 2024~~).

2. Outline of production and minimum requirements for conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

For killed or live vaccines, the bacterial strain should be an organism as closely related to currently circulating field strains as possible. It should be carefully chosen from cases of severe clinical disease, and virulence and antigen production should be assessed. It is best to evaluate a panel of potential strains in this way before testing the final selection. The final vaccinal strain should be identified by historical records and characterised by stable phenotypic and/or genetic markers, preferably using whole genome sequencing. Live vaccinal strains should be marked by stable characters allowing easy distinction from wild strains. Markers such as resistance to antimicrobials, for example rifampicin, or auxotrophism may be used. Attenuation of virulence should be stable and preferably obtained by two independent defined mutations. The stability of live vaccine strains can be verified by regular checks using whole genome sequencing.

Live fowl typhoid vaccine is a suspension of suitably attenuated living organisms of a rough strain of *S. Gallinarum*, e.g. 9R. The organisms in the vaccine give the biochemical reactions characteristic of *S. Gallinarum*. ~~Colonies of a 24 hour culture prepared from the vaccine on nutrient agar plates are rough when examined by the acriflavine slide test.~~ The culture should not produce any smooth colonies or contain the somatic antigens characteristic of the smooth forms of *S. Gallinarum*.

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

i) Sterility and purity

The vaccine strain must be checked as follows:

- a) Staining of a smear of bacterial suspension on a glass slide using Gram stain.
- b) Homogeneity of culture on non-selective media.
- c) Metabolic requirements as indicated by biochemical tests.
- d) Detection of phenotypic and/or genetic markers.
- e) Agglutination with specific antiserum.
- f) The vaccine culture and any adjuvants, preservatives or other materials must be microbiologically sterile and non-toxic at the concentrations used.

ii) Safety

The LD₅₀ (50% lethal dose) or ID₅₀ (50% infectious dose) may be determined in chickens or, preferably, signs of more mild adverse reactions should be checked in the target species. Ten times the field dose of live vaccine or twice the dose for killed vaccines must be given to the target species at the recommended age and by the recommended route. The animals are observed for absence of adverse reactions. Stability and non-reversion to virulence after serial passages in susceptible species should be shown for live vaccines. It is also necessary to consider repeat vaccination. Live vaccine should be shown not to persist for long periods in vaccinated animals or be transmitted to meat or eggs that may be consumed, and the method of application should not present a hazard to operators. In the case of *S. Gallinarum* vaccine at least six healthy, susceptible (preferably specific pathogen free [SPF]) chickens, 8–16 weeks of age, are each injected subcutaneously with a ten-fold dose of vaccine, and are observed for at least 7 days; no local or systemic reaction should develop.

iii) Efficacy

Laboratory experiments and field trials should be used to show that the vaccine is effective. The laboratory experiments consist of vaccination–challenge tests in the target species at the recommended dose and age. The efficacy data can also be used as the basis for a batch potency test. Field trials are more difficult to undertake with respect to testing efficacy because of difficulties with standardising the challenge and providing appropriate controls. In the case of *S. Gallinarum* 9R vaccine or similar vaccines, at least fifteen healthy chickens, 8–16 weeks of age, of a brown layer hybrid breed, and taken from a stock that is free from *S. Pullorum* infection, are each injected subcutaneously with a quantity of vaccine corresponding to one field dose, i.e. 5×10^7 viable organisms. After an interval of 21–28 days, the vaccinated chickens and an equal number of similar unvaccinated chickens are deprived of food for approximately 18 hours. The chickens are then challenged by oral administration of 1 ml of a broth suspension containing 5×10^7 organisms of a virulent strain of *S. Gallinarum* mixed with 300 mg of a powder consisting of chalk (40%), light kaolin (43%) and magnesium trisilicate (17%). All the chickens are observed for 14–21 days. The vaccine passes the test if at the end of this period the number of surviving vaccinated chickens that show no macroscopic lesions of fowl typhoid at post-mortem exceeds by eight or more the number of similarly defined control chickens.

iv) Environmental aspects

Live vaccine strains should be tested for their ability to persist in the environment and infect non-target species such as rodents and wild birds that are likely to be exposed. Prolonged survival of some live vaccines in faeces, litter or dust may present an unacceptable environmental hazard when the material is removed from the animal houses. Live vaccines should not be used in commercial laying flocks during lay.

2.2. Method of manufacture

2.2.1. Procedure

The seed culture is propagated and maintained using suitable media for growth of *Salmonella*. The media used should not contain serum or animal tissues, unless permitted by national regulations. Culture may be on solid medium, in Roux flasks, or in liquid medium, in which case large-scale fermentation equipment may be used. Iron limitation or low temperature incubation on minimal media may enhance LPS antigen production by the vaccine strain. In the case of *S. Gallinarum* (9R), the vaccine may be prepared by inoculation of a suitable medium, such as peptone broth, with a fresh culture of *S. Gallinarum* (9R) and incubation at 37°C for 24 hours, with agitation. The organisms are harvested by sedimentation or centrifugation.

Alternatively the organisms may be grown on and harvested from a solid medium, such as nutrient agar. In either case, the suspension is diluted in PBS solution, pH 7.0, and may be freeze-dried. The dose used per bird is between 5×10^6 and 5×10^7 organisms.

Vaccine must be produced in suitable clean rooms to which only approved personnel have access. Care must be taken to avoid cross-contamination between areas where live organisms are processed and other areas. Contamination from operators and/or the environment must be avoided and vaccine preparation should take place in a separate area from diagnostic culture work. Operators must not work with vaccine whilst ill and must not be subject to immunosuppressive conditions or medications. Personnel must be provided with protective clothing in production areas and in animal rooms.

Seed-lot cultures are prepared from the primary seed-lot, and the number of passages is dependent on the validation of the process. The vaccine may be prepared by inoculation of a suitable medium, such as nutrient broth, with a fresh culture and incubation on a shaker at 37°C for 24 hours, with or without aeration. The organisms are harvested by sedimentation or centrifugation. Alternatively, the organisms may be grown on and harvested from a solid medium, such as nutrient agar. In the case of live vaccines, the suspension is diluted in PBS, pH 7.0, and may be freeze-dried.

The time of inactivation of dead vaccines should be at least 33% more than that taken to reduce the viable number to an undetectable level. The inactivation process must be applied to the whole volume of the vaccine cell harvest.

Preservatives, excipient for lyophilisation, stabiliser for multi-dose containers or other substances added to or combined with a vaccinal preparation must have no deleterious effect on the immunising potency of the product.

2.2.2. Requirements for substrates and media

All chemicals and growth media used should be guaranteed to be fit for purpose and checked by the use of suitable controls.

2.2.3. In-process controls

The following points require attention:

- i) Visual control of the suspension, homogeneity by Gram stain, culture on non-selective medium.
- ii) Slide agglutination with specific antisera.
- iii) Titration of bacteria by turbidimetry and/or plate count.
- iv) Test of effective inactivation (killed vaccine) by plating on non-selective medium or use of a medium that gives optimum chance of recovery, e.g. production medium with neutralisation of the inactivating compound.
- v) Titration of viable bacteria (living vaccine) before and after lyophilisation.

2.2.4. Final product batch tests

- i) Sterility/purity

Tests for sterility and freedom from contamination of biological materials intended for veterinary use are found in chapter 1.1.9 of this *Terrestrial Manual*.

- ii) Safety

A laboratory test that has previously shown a correlation with safety in the target species may be used to determine the absence of deleterious effects on vaccinated animals. Each batch should be tested in the target species at the recommended age and route, using at least twice the field dose for killed vaccines and ten times the dose for live vaccines. Observations are made on any adverse effects on the demeanour and health of the vaccinated animals and an assessment may be made of tissue reactions at the injection site.

Safety tests in target animals are not required by many regulatory authorities for the release of each batch. Where required, standard procedures are generally conducted using fewer animals than are used in the safety tests required for the relevant regulatory approval.

- iii) Batch potency

Potency is tested using vaccination–challenge assay in chickens and/or other species, including (if practicable) any other target species and immunological response in target species.

2.3. Requirements for authorisation

2.3.1. Safety requirements

Certain killed vaccines may occasionally cause reactions in vaccinated animals because of their LPS content or the adjuvant used, and likewise live vaccines should be used with caution in animals that are not completely healthy at the time of vaccination. It is often necessary, however, to vaccinate flocks in the face of clinical fowl typhoid. Vaccines may also cause swelling at the site of injection, particularly if an oil-emulsion adjuvant is used.

i) Target and non-target animal safety

Killed vaccines are assessed in a double-dose test, and live vaccines are assessed in a test using ten times the dose, ideally in the target species. Live vaccines should be proven to be harmless in relevant non-target species that could be exposed to vaccine shed by vaccinated animals. As *S. Gallinarum* and *S. Pullorum* are host specific, non-target species are less of a concern.

ii) Reversion to virulence for attenuated/live vaccines

Live vaccines shall be shown in replication tests in target species to not revert to virulent strains during a suitably large number of replications. Mutations, especially undefined mutations, should be shown to be stable, and checks on stability can be made by whole genome sequencing. It is recommended that live vaccines that contain *Salmonella* serovars that are not endemic in a particular region should not be used for control of other serovars (van Immerseel *et al.*, 2013). Special care should also be taken to ensure that attenuated vaccines are not incompletely attenuated or contaminated with seed organisms.

iii) Environmental consideration

Live vaccines should not be able to replicate in the environment or persist for more than a short period.

2.3.2. Efficacy requirements

i) For animal production

The duration of immunity is likely to vary considerably between products, vaccination regimes and individual vaccinated animals. The vaccine should provide protection throughout the laying period, and this can be measured by potency (efficacy) tests at stages during lay. A booster dose during lay may be required, but live vaccines should not be used during lay in flocks providing eggs for human consumption.

Immunity to *Salmonella* is normally serovar or serogroup specific. Consultation among colleagues suggests that most killed vaccines will provide some protection for 6 months, while some live vaccines given by injection may elicit stronger immunity, which may persist for 1 year or more. Orally administered vaccines may produce more variable protection, particularly in the case of labile vaccines that are administered in drinking water. Vaccination of day old chicks by coarse spray may be beneficial where there is early challenge, and programmes that combine live and killed vaccines may provide superior protection. It should be remembered however, that a strong challenge such as that associated with continually occupied farms or infected wild birds and mite populations may overwhelm vaccinal immunity and commercial live vaccines may be attenuated to reduce environmental survival in a way that reduces the immune response. There may also be problems with ensuring accuracy of injection with killed and live injectable vaccines. The *Salmonella* vaccines are intended to limit the extent of clinical disease in poultry, and also to reduce the risk of introduction of infection to flocks. If possible, the potency test should relate to the efficacy of the vaccine in the target species, and suitable criteria should be applied for passing batches. It may be possible to assess killed and injected vaccines by the O-H antibody response produced, although it should be remembered that serum antibodies are only part of the host's protective mechanism against *Salmonella*. Alternatively, the potency of the vaccine may be assessed by its effect on challenged vaccinated animals compared quantitatively and statistically with unvaccinated controls.

ii) For control and eradication

Vaccines for *Salmonella* are not capable of eradicating infection from flocks but can increase the threshold for infection, reduce the level of shedding of the organism and reduce vertical transmission in poultry that results in contamination of hatching or table eggs. Vaccination

is therefore an aid to other eradication and control measures such as culling, all in-all out production, biosecurity and farm hygiene.

2.3.3. Stability

Information is lacking on the stability of killed vaccines. Stability is affected by storage conditions and by the presence of contaminating microorganisms growing in the product. Chemicals with antimicrobial activity, such as thiomersal, phenol or crystal violet, are often included as preservatives in killed bacterial vaccines. The stability is assessed by potency tests repeated at appropriate time intervals. The stability of live vaccines can be assessed by performing counts of the number of viable organisms repeated at appropriate time intervals, and genotyping tests to identify genetic changes during fermentation production.

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NB: At the time of publication (2018) there were no WOA Reference Laboratories for fowl typhoid and pullorum disease (please consult the WOA Web site: <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>).

NB: FIRST ADOPTED IN 1991. MOST RECENT UPDATES ADOPTED IN 2018.

Appendix 1

Fowl typhoid and Pullorum disease Intended purpose of test: population freedom from infection

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Bacterial isolation</u> ☒ ☒ ☒	<u>Live birds: cloacal swabs, fresh faeces</u> <u>Carcasses: different organ samples, e.g. liver, ileocaecal junction, ovaries, oviduct, spleen, egg material, embryos</u> <u>Environmental samples: fluff, debris, waste</u>	<u>Accuracy depends on media selection, with selenite cystine and Rappaport–Vassiliadis showing better performance</u>	<u>Entire flocks or representative groups, mainly breeding chickens of all ages</u>	<u>See reference</u>	<u>1. Considered the gold standard for confirming active infections</u> <u>2. Allows characterisation and further antibiotic susceptibility testing</u>	<u>1. Time-consuming (48+ hours for colony growth)</u> <u>2. Requires specific enrichment media (e.g. selenite cystine, Rappaport–Vassiliadis) for optimal sensitivity</u> <u>3. Swarm agar cannot be used because <i>S. Gallinarum</i>/Pullorum does not possess flagellar antigen and are non-motile</u> <u>4. Colonies of <i>S. Gallinarum</i>/Pullorum are smaller and slower growing compared with other <i>Salmonella</i> serovars</u>	<u>Proux <i>et al.</i> (2002)</u> <u>ISO 6579-1</u> <u>WOAH <i>Terrestrial Manual</i></u>
<u>PCR</u> ☒ ☒ ☐	<u><i>Salmonella</i> isolates</u> <u>Tissue samples</u>	<u>100% specificity</u>	<u>Entire flocks or representative groups, mainly breeding chickens of all ages</u>	<u>See reference</u>	<u>1. Rapid (results in hours), high specificity, and sensitivity</u> <u>2. Multiplex PCR can differentiate <i>S. Gallinarum</i> and Pullorum</u> <u>3. Low detection limit: 10 CFU or 5.85 pg/μl of DNA</u>	<u>1. Requires specialised equipment and technical expertise</u> <u>2. May not distinguish live from dead bacteria</u>	<u>Xiong <i>et al.</i> (2016)</u> <u>Cocciolo <i>et al.</i> (2020)</u> <u>Song <i>et al.</i> (2023)</u> <u>Wang <i>et al.</i> (2025)</u>
<u>Whole blood agglutination</u> ☒ ☒ ☐	<u>Blood</u>	<u>No data available</u>	<u>Entire flocks or representative groups, mainly breeding chickens of all ages</u>	<u>See reference</u>	<u>1. Rapid procedure: the test can be performed directly on whole blood without the need for serum separation</u> <u>2. Field-friendly: ideal for field use or in low-resource settings</u> <u>3. Cost-effective: reduces material and equipment costs</u>	<u>1. May have reduced sensitivity compared to serum-based tests, especially if antibody levels are low</u> <u>2. Cellular components in whole blood can interfere with agglutination and affect result interpretation</u>	<u>WOAH <i>Terrestrial Manual</i></u> <u>Thain <i>et al.</i> (1981)</u>
<u>Rapid serum agglutination</u> ☒ ☒ ☐	<u>Sera</u>	<u>Accuracy improved by diluting serum to 1:4 to reduce false positives</u>	<u>Entire flocks or representative groups, mainly breeding chickens of all ages</u>	<u>See reference</u>	<u>1. Low-cost and quick (minutes)</u> <u>2. Practical for flock screening</u> <u>3. Higher sensitivity and specificity than whole blood agglutination</u>	<u>1. Requires basic equipment to separate serum</u> <u>2. Subjective interpretation of results</u> <u>3. False positives, especially undiluted serum</u>	<u>Proux <i>et al.</i> (2002)</u> <u>Ter Veen <i>et al.</i> (2022)</u>

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Tube agglutination</u> ☒ ☒ ☐	<u>Sera</u>	<u>Accuracy is high when used to confirm results of rapid serum agglutination</u>	<u>Entire flocks or representative groups, mainly breeding chickens of all ages</u>	<u>See reference</u>	<u>1. More specific and quantitative than rapid serum agglutination or whole blood agglutination</u> <u>2. Allows titration for antibody levels; reliable for confirmatory diagnostics</u> <u>3.Reduces chance of false positives</u>	<u>1. Requires laboratory equipment</u> <u>2. More labour-intensive and time-consuming (incubation overnight)</u> <u>3. Not as field friendly as whole blood or rapid serum agglutination</u>	<u>WOAH <i>Terrestrial Manual</i> Williams & Whittemore (1971)</u>
<u>Micro-agglutination</u> ☒ ☒ ☐	<u>Sera</u>	<u>Demonstrated accuracy and sensitivity in routine poultry screening</u>	<u>Entire flocks or representative groups, mainly breeding chickens of all ages</u>	<u>See reference</u>	<u>1. Enables testing with small sample volumes (suitable for large-scale screening)</u> <u>2. Highly sensitive and more quantitative than rapid serum/slide methods</u> <u>3. Suitable for automation</u>	<u>1. Specialised equipment (microplates) required</u> <u>2. Technique initially developed for research laboratories</u>	<u>Williams & Whittemore (1971)</u>
<u>ELISA</u> ☒ ☐ ☐	<u>Sera</u>	<u>100% sensitivity for S. Gallinarum</u>	<u>Entire flocks or representative groups, mainly breeding chickens of all ages</u>	<u>See reference</u>	<u>1. High-throughput and quantitative</u> <u>2. Specific for S. Gallinarum/Pullorum when using LPS or soluble protein antigens</u>	<u>1. Cross-reactivity with S. Enteritidis if antigens are not optimised</u> <u>2. Requires standardised reagents</u>	<u>Proux <i>et al.</i> (2002)</u> <u>Oliveira <i>et al.</i> (2004)</u>

Appendix 2

Fowl typhoid and Pullorum disease

Intended purpose of test: individual animal freedom from infection prior to movement

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Bacterial isolation</u> ☒ ☒ ☒	<u>Live birds: cloacal swabs, fresh faeces</u> <u>Carcasses: different organ samples, e.g. liver, ileocaecal junction, ovaries, oviduct, spleen, egg material, embryos</u> <u>Environmental samples: fluff, debris, waste</u>	<u>Accuracy depends on media selection, with selenite cystine and Rappaport–Vassiliadis showing better performance</u>	<u>Individual birds, mainly breeding stock or layers, generally adults (>4 weeks)</u>	<u>See reference</u>	<u>1. Considered the gold standard for confirming active infections</u> <u>2. Allows characterisation and further antibiotic susceptibility testing</u>	<u>1. Time-consuming (48+ hours for colony growth)</u> <u>2. Requires specific enrichment media (e.g. selenite cystine, Rappaport–Vassiliadis) for optimal sensitivity</u> <u>3. Swarm agar cannot be used because <i>S. Gallinarum</i>/Pullorum does not possess flagellar antigen and are non-motile</u> <u>4. Colonies of <i>S. Gallinarum</i>/Pullorum are smaller and slower growing compared with other <i>Salmonella</i> serovars</u>	<u>Proux <i>et al.</i> (2002) ISO 6579-1</u> <u>WOAH <i>Terrestrial Manual</i></u>
<u>PCR</u> ☒ ☒ ☐	<u><i>Salmonella</i> isolates</u> <u>Tissue samples</u>	<u>100% specificity</u>	<u>Individual birds, mainly breeding stock or layers, generally adults (>4 weeks)</u>	<u>See reference</u>	<u>1. Rapid (results in hours), high specificity, and sensitivity</u> <u>2. Multiplex PCR can differentiate <i>S. Gallinarum</i> and Pullorum</u> <u>3. Low detection limit: 10 CFU or 5.85 pg/ul of DNA</u>	<u>1. Requires specialised equipment and technical expertise</u> <u>2. May not distinguish live from dead bacteria</u>	<u>Xiong <i>et al.</i> (2016)</u> <u>Cocciolo <i>et al.</i> (2020)</u> <u>Song <i>et al.</i> (2023)</u> <u>Wang <i>et al.</i> (2025)</u>

Appendix 3

Fowl typhoid and Pullorum disease Intended purpose of test: contribute to eradication policies

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Bacterial isolation</u> ☒ ☒ ☒	<u>Live birds: cloacal swabs, fresh faeces</u> <u>Carcasses: different organ samples, e.g. liver, ileocaecal junction, ovaries, oviduct, spleen, egg material, embryos</u> <u>Environmental samples: fluff, debris, waste</u>	<u>Accuracy depends on media selection, with selenite cystine and Rappaport–Vassiliadis showing better performance</u>	<u>All birds in infected/suspect flocks of all ages and relevant species</u>	<u>See reference</u>	<u>1. Considered the gold standard for confirming active infections</u> <u>2. Allows characterisation and further antibiotic susceptibility testing</u>	<u>1. Time-consuming (48+ hours for colony growth)</u> <u>2. Requires specific enrichment media (e.g. selenite cystine, Rappaport–Vassiliadis) for optimal sensitivity</u> <u>3. Swarm agar cannot be used because <i>S. Gallinarum</i>/<i>Pullorum</i> does not possess flagellar antigen and are non-motile</u> <u>4. Colonies of <i>S. Gallinarum</i>/<i>Pullorum</i> are smaller and slower growing compared with other <i>Salmonella</i> serovars</u>	<u>Proux <i>et al.</i> (2002)</u> <u>ISO 6579-1</u> <u>WOAH <i>Terrestrial Manual</i></u>
<u>PCR</u> ☒ ☒ ☐	<u><i>Salmonella</i> isolates</u> <u>Tissue samples</u>	<u>100% specificity</u>	<u>All birds in infected/suspect flocks of all ages and relevant species</u>	<u>See reference</u>	<u>1. Rapid (results in hours), high specificity, and sensitivity</u> <u>2. Multiplex PCR can differentiate <i>S. Gallinarum</i> and <i>Pullorum</i></u> <u>3. Low detection limit: 10 CFU or 5.85 pg/μl of DNA</u>	<u>1. Requires specialised equipment and technical expertise</u> <u>2. May not distinguish live from dead bacteria</u>	<u>Xiong <i>et al.</i> (2016)</u> <u>Cocciolo <i>et al.</i> (2020)</u> <u>Song <i>et al.</i> (2023)</u> <u>Wang <i>et al.</i> (2025)</u>
<u>Whole blood agglutination</u> ☒ ☒ ☒	<u>Blood</u>	<u>No data available</u>	<u>All birds in infected/suspect flocks of all ages and relevant species</u>	<u>See reference</u>	<u>1. Rapid procedure: the test can be performed directly on whole blood without the need for serum separation</u> <u>2. Field-friendly: ideal for field use or in low-resource settings</u> <u>3. Cost-effective: reduces material and equipment costs</u>	<u>1. May have reduced sensitivity compared to serum-based tests, especially if antibody levels are low</u> <u>2. Cellular components in whole blood can interfere with agglutination and affect result interpretation</u>	<u>WOAH <i>Terrestrial Manual</i></u> <u>Thain <i>et al.</i> (1981)</u>

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Rapid serum agglutination</u> ☒ ☐ ☐	<u>Sera</u>	<u>Accuracy improved by diluting serum to 1:4 to reduce false positives</u>	<u>All birds in infected/suspect flocks of all ages and relevant species</u>	<u>See reference</u>	<u>1. Low-cost and quick (minutes)</u> <u>2. Practical for flock screening</u> <u>3. Higher sensitivity and specificity than whole blood agglutination.</u>	<u>1. Requires basic equipment to separate serum</u> <u>2. Subjective interpretation of results</u> <u>3. False positives, especially undiluted serum</u>	<u>Proux <i>et al.</i> (2002)</u> <u>Ter Veen <i>et al.</i> (2022)</u>
<u>Tube agglutination</u> ☒ ☐ ☐	<u>Sera</u>	<u>Accuracy is high when used to confirm results of rapid serum agglutination</u>	<u>All birds in infected/suspect flocks of all ages and relevant species</u>	<u>See reference</u>	<u>1. More specific and quantitative than rapid serum agglutination or whole blood agglutination</u> <u>2. Allows titration for antibody levels; reliable for confirmatory diagnostics</u> <u>3. Reduces chance of false positives</u>	<u>1. Requires laboratory equipment</u> <u>2. More labour-intensive and time-consuming (incubation overnight)</u> <u>3. Not as field friendly as whole blood or rapid serum agglutination</u>	<u>WOAH <i>Terrestrial Manual</i> Williams & Whittemore (1971)</u>
<u>Micro-Agglutination</u> ☒ ☐ ☐	<u>Sera</u>	<u>Demonstrated accuracy and sensitivity in routine poultry screening</u>	<u>All birds in infected/suspect flocks of all ages and relevant species</u>	<u>See reference</u>	<u>1. Enables testing with small sample volumes (suitable for large-scale screening)</u> <u>2. Highly sensitive and more quantitative than rapid serum/slide methods</u> <u>3. Suitable for automation</u>	<u>1. Specialised equipment (microplates) required</u> <u>2. Technique initially developed for research laboratories</u>	<u>Williams & Whittemore (1971)</u>
<u>ELISA</u> ☒ ☐ ☐	<u>Sera</u>	<u>100% sensitivity for <i>S. Gallinarum</i></u>	<u>All birds in infected/suspect flocks of all ages and relevant species</u>	<u>See reference</u>	<u>1. High-throughput and quantitative</u> <u>2. Specific for <i>S. Gallinarum</i>/Pullorum when using LPS or soluble protein antigens</u>	<u>1. Cross-reactivity with <i>S. Enteritidis</i> if antigens are not optimised</u> <u>2. Requires standardised reagents</u>	<u>Proux <i>et al.</i> (2002)</u> <u>Oliveira <i>et al.</i> (2004)</u>

Appendix 4

Fowl typhoid and Pullorum disease Intended purpose of test: confirmation of clinical cases

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Bacterial isolation</u> ☒ ☒ ☒	<u>Live birds: cloacal swabs, fresh faeces</u> <u>Carcasses: different organ samples, e.g. liver, ileocaecal junction, ovaries, oviduct, spleen, egg material, embryos</u> <u>Environmental samples: fluff, debris, waste</u>	<u>Accuracy depends on media selection, with selenite cystine and Rappaport–Vassiliadis showing better performance</u>	<u>Clinically suspect birds (young or adult)</u>	<u>See reference</u>	<u>1. Considered the gold standard for confirming active infections</u> <u>2. Allows characterisation and further antibiotic susceptibility testing</u>	<u>1. Time-consuming (48+ hours for colony growth)</u> <u>2. Requires specific enrichment media (e.g. selenite cystine, Rappaport–Vassiliadis) for optimal sensitivity</u> <u>3. Swarm agar cannot be used because <i>S. Gallinarum</i>/Pullorum does not possess flagellar antigen and are non-motile</u> <u>4. Colonies of <i>S. Gallinarum</i>/Pullorum are smaller and slower growing compared with other <i>Salmonella</i> serovars</u>	<u>Proux <i>et al.</i> (2002) ISO 6579-1</u> <u>WOAH <i>Terrestrial Manual</i></u>
<u>PCR</u> ☒ ☒ ☐	<u><i>Salmonella</i> isolates</u> <u>Tissue samples</u>	<u>100% specificity</u>	<u>Clinically suspect birds (young or adult)</u>	<u>See reference</u>	<u>1. Rapid (results in hours), high specificity, and sensitivity</u> <u>2. Multiplex PCR can differentiate <i>S. Gallinarum</i> and Pullorum</u> <u>3. Low detection limit: 10 CFU or 5.85 pg/ul of DNA</u>	<u>1. Requires specialised equipment and technical expertise</u> <u>2. May not distinguish live from dead bacteria</u>	<u>Xiong <i>et al.</i> (2016)</u> <u>Cocciolo <i>et al.</i> (2020)</u> <u>Song <i>et al.</i> (2023)</u> <u>Wang <i>et al.</i> (2025)</u>

Appendix 5

Fowl typhoid and Pullorum disease Intended purpose of test: prevalence of infection – surveillance

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Bacterial isolation</u> ☒ ☒ ☒	<u>Live birds: cloacal swabs, fresh faeces</u> <u>Carcasses: different organ samples, e.g. liver, ileocaecal junction, ovaries, oviduct, spleen, egg material, embryos</u> <u>Environmental samples: fluff, debris, waste</u>	<u>Accuracy depends on media selection, with selenite cystine and Rappaport–Vassiliadis showing better performance</u>	<u>Representative sampling in commercial flocks, mainly chickens</u>	<u>See reference</u>	<u>1. Considered the gold standard for confirming active infections</u> <u>2. Allows characterisation and further antibiotic susceptibility testing</u>	<u>1. Time-consuming (48+ hours for colony growth)</u> <u>2. Requires specific enrichment media (e.g. selenite cystine, Rappaport–Vassiliadis) for optimal sensitivity</u> <u>3. Swarm agar cannot be used because <i>S. Gallinarum</i>/<i>Pullorum</i> does not possess flagellar antigen and are non-motile</u> <u>4. Colonies of <i>S. Gallinarum</i>/<i>Pullorum</i> are smaller and slower-growing compared to other <i>Salmonella</i> serovars</u>	<u>Proux <i>et al.</i> (2002)</u> <u>ISO 6579-1</u> <u>WOAH <i>Terrestrial Manual</i></u>
<u>PCR</u> ☒ ☒ ☐	<u><i>Salmonella</i> isolates</u> <u>Tissue samples</u>	<u>100% specificity</u>	<u>Representative sampling in commercial flocks, mainly chickens</u>	<u>See reference</u>	<u>1. Rapid (results in hours), high specificity, and sensitivity</u> <u>2. Multiplex PCR can differentiate <i>S. Gallinarum</i> and <i>Pullorum</i></u> <u>3. Low detection limit: 10 CFU or 5.85 pg/ul of DNA</u>	<u>1. Requires specialised equipment and technical expertise</u> <u>2. May not distinguish live from dead bacteria</u>	<u>Xiong <i>et al.</i> (2016)</u> <u>Cocciolo <i>et al.</i> (2020)</u> <u>Song <i>et al.</i> (2023)</u> <u>Wang <i>et al.</i> (2025)</u>
<u>Whole blood agglutination</u> ☒ ☐ ☐	<u>Blood</u>	<u>No data available</u>	<u>Representative sampling in commercial flocks, mainly chickens</u>	<u>See reference</u>	<u>1. Rapid procedure: the test can be performed directly on whole blood without the need for serum separation</u> <u>2. Field-friendly: ideal for field use or in low-resource settings</u> <u>3. Cost-effective: reduces material and equipment costs</u>	<u>1. May have reduced sensitivity compared to serum-based tests especially if antibody levels are low</u> <u>2. Cellular components in whole blood can interfere with agglutination and affect result interpretation</u>	<u>WOAH <i>Terrestrial Manual</i></u> <u>Thain <i>et al.</i> (1981)</u>
<u>Rapid serum agglutination</u> ☒ ☐ ☐	<u>Sera</u>	<u>Accuracy improved by diluting serum to 1:4 to reduce false positives</u>	<u>Representative sampling in commercial flocks, mainly chickens</u>	<u>See reference</u>	<u>1. Low-cost and quick (minutes)</u> <u>2. Practical for flock screening</u> <u>3. Higher sensitivity and specificity than whole blood agglutination</u>	<u>1. Requires basic equipment to separate serum</u> <u>2. Subjective interpretation of results</u> <u>2. False positives, especially undiluted serum</u>	<u>Proux <i>et al.</i> (2002)</u> <u>Ter Veen <i>et al.</i> (2022)</u>

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Tube agglutination</u> ☒ ☒ ☐	<u>Sera</u>	<u>accuracy is high when used to confirm results of rapid serum agglutination</u>	<u>Representative sampling in commercial flocks, mainly chickens</u>	<u>See reference</u>	<u>1. More specific and quantitative than rapid serum agglutination or whole blood agglutination</u> <u>2. Allows titration for antibody levels; reliable for confirmatory diagnostics</u> <u>3.Reduces chance of false positives</u>	<u>1. Requires laboratory equipment</u> <u>2. More labour-intensive and time-consuming (incubation overnight)</u> <u>3. Not as field friendly as whole blood or rapid serum agglutination</u>	<u>WOAH <i>Terrestrial Manual</i> Williams & Whittemore (1971)</u>
<u>Micro-agglutination</u> ☒ ☒ ☐	<u>Sera</u>	<u>Demonstrated accuracy and sensitivity in routine poultry screening</u>	<u>Representative sampling in commercial flocks, mainly chickens</u>	<u>See reference</u>	<u>1.Enables testing with small sample volumes (suitable for large-scale screening)</u> <u>2. Highly sensitive and more quantitative than rapid serum/slide methods</u> <u>3. Suitable for automation</u>	<u>1.Specialised equipment (microplates) required</u> <u>2. Technique initially developed for research laboratories</u>	<u>Williams & Whittemore (1971)</u>
<u>ELISA</u> ☒ ☐ ☐	<u>Sera</u>	<u>100% sensitivity for <i>S. Gallinarum</i></u>	<u>Representative sampling in commercial flocks, mainly chickens</u>	<u>See reference</u>	<u>1. High-throughput and quantitative</u> <u>2. Specific for <i>S. Gallinarum</i>/Pullorum when using LPS or soluble protein antigens</u>	<u>1. Cross-reactivity with <i>S. Enteritidis</i> if antigens are not optimised</u> <u>2. Requires standardised reagents</u>	<u>Proux <i>et al.</i> (2002)</u> <u>Oliveira <i>et al.</i> (2004)</u>

Appendix 6

Fowl typhoid and Pullorum disease

Intended purpose of test: immune status in individual animals or populations post-vaccination

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Whole blood agglutination</u> ☒ ☒ ☐	<u>Blood</u>	<u>No data available</u>	<u>Vaccinated flocks, mainly mature birds</u>	<u>See reference</u>	<u>1. Rapid procedure: the test can be performed directly on whole blood without the need for serum separation</u> <u>2. Field-friendly: ideal for field use or in low-resource settings</u> <u>3. Cost-effective: reduces material and equipment costs</u>	<u>1. May have reduced sensitivity compared to serum-based tests, especially if antibody levels are low</u> <u>2. Cellular components in whole blood can interfere with agglutination and affect result interpretation</u>	<u>WOAH Terrestrial Manual</u> <u>Thain et al. (1981)</u>
<u>Rapid serum agglutination</u> ☒ ☒ ☐	<u>Sera</u>	<u>Accuracy improved by diluting serum to 1:4 to reduce false positives</u>	<u>Vaccinated flocks, mainly mature birds</u>	<u>See reference</u>	<u>1. Low-cost and quick (minutes)</u> <u>2. Practical for flock screening</u> <u>3. Higher sensitivity and specificity than whole blood agglutination</u>	<u>1. Requires basic equipment to separate serum</u> <u>2. Subjective interpretation of results</u> <u>2. false positives, especially undiluted serum</u>	<u>Proux et al. (2002)</u> <u>Ter Veen et al. (2022)</u>
<u>Tube agglutination</u> ☒ ☒ ☒	<u>Sera</u>	<u>accuracy is high when used to confirm results of rapid serum agglutination</u>	<u>Vaccinated flocks, mainly mature birds</u>	<u>See reference</u>	<u>1. More specific and quantitative than rapid serum agglutination or whole blood agglutination</u> <u>2. Allows titration for antibody levels: reliable for confirmatory diagnostics</u> <u>3. Reduces chance of false positives</u>	<u>1. Requires laboratory equipment</u> <u>2. More labour-intensive and time-consuming (incubation overnight)</u> <u>3. Not as field friendly as whole blood or rapid serum agglutination</u>	<u>WOAH Terrestrial Manual</u> <u>Williams & Whittemore (1971)</u>
<u>Micro-agglutination</u> ☒ ☒ ☒	<u>Sera</u>	<u>Demonstrated accuracy and sensitivity in routine poultry screening</u>	<u>Vaccinated flocks, mainly mature birds</u>	<u>See reference</u>	<u>1. Enables testing with small sample volumes (suitable for large-scale screening)</u> <u>2. Highly sensitive and more quantitative than rapid serum/slide methods</u> <u>3. Suitable for automation</u>	<u>1. Specialised equipment (microplates) required</u> <u>2. Technique initially developed for research laboratories</u>	<u>Williams & Whittemore (1971)</u>
<u>ELISA</u> ☒ ☒ ☐	<u>Sera</u>	<u>100% sensitivity for S. Gallinarum</u>	<u>Vaccinated flocks, mainly mature birds</u>	<u>See reference</u>	<u>1. High-throughput and quantitative</u> <u>2. Specific for S. Gallinarum/Pullorum when using LPS or soluble protein antigens</u>	<u>1. Cross-reactivity with S. Enteritidis if antigens are not optimised</u> <u>2. Requires standardised reagents.</u>	<u>Proux et al. (2002)</u> <u>Oliveira et al. (2004)</u>

MEETING OF THE BIOLOGICAL STANDARDS COMMISSION

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CHAPTER 3.4.4.

BOVINE GENITAL CAMPYLOBACTERIOSIS

SUMMARY

Description of the disease: Bovine genital campylobacteriosis (BGC) is a venereal disease also known as bovine venereal campylobacteriosis. The causal agent of this sexually transmissible disease is *Campylobacter fetus* subsp. *venerealis*, one of three subspecies of *C. fetus*. Bovine infections with *C. fetus* subsp. *venerealis* are associated with infertility, early embryonic death, and abortion with considerable economic losses. Bovine infections with *C. fetus* subsp. *fetus* are associated with abortion and have a more sporadic occurrence.

Campylobacter fetus subsp. *venerealis* has a pronounced tropism for the male and female genital systems of cattle. Bacterial transmission takes place mainly during natural mating. The disease may also spread through artificial insemination with semen from infected bulls.

Detection of the agent: Samples taken from bulls, cows or aborted fetuses can be analysed for the presence of the causal organism. The organism is a thin Gram-negative curved rod that may form S-shapes, flying seagull silhouette shapes and spirals, and can be cultured on selective media at 37°C after ~~at least 2–3~~ days in a microaerobic atmosphere. Confirmation of organism and discrimination between the *C. fetus* subspecies can be achieved using biochemical or molecular methods, such as polymerase chain reaction and whole genome sequencing. Immunofluorescence may also be used to identify the organism, but it will not differentiate between different subspecies.

A monoclonal antibody-based capture enzyme-linked immunosorbent assay (ELISA) may also be used to detect *C. fetus* from Clarke's transport enrichment medium. Like immunofluorescence, the ELISA cannot differentiate adequately between the two *C. fetus* subspecies and is limited to a screening method, with traditional cultural methods to confirm identification of any positive ELISA result. However, compared with immunofluorescence, it has the advantage of higher sensitivity and specificity for *C. fetus* identification and much higher throughput for both negative and positive results where larger volume testing is conducted.

Serological tests: ELISA can be used for evaluating herd immunity but is not suitable for diagnosis of the infection in individual animals. This test cannot differentiate between infections caused by the two subspecies *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *fetus*.

Requirements for vaccines: While there are commercial vaccines available, and autologous vaccines based on isolated strains from farms can be used, there is a lack of scientific evidence overall of the effectiveness of vaccination.

A. INTRODUCTION

1. Description and impact of the disease

Bovine genital campylobacteriosis (BGC, also known as bovine venereal campylobacteriosis) is a venereal disease characterised by infertility, early embryonic death, and abortion in cattle. The causal agent of this sexually transmissible disease is *Campylobacter fetus* subsp. *venerealis* (Cfv). It can be isolated from the genital tract of cattle (e.g. preputial smegma, vaginal mucus) or internal organs of aborted fetuses and causes fertility problems with considerable economic losses. Bulls are important reservoirs and should therefore be targeted for diagnostic testing and disease control. *Campylobacter fetus* subsp. *fetus* (Cff) can be recovered from the intestinal tract of cattle and other animal species (Garcia *et al.*, 1983); it can be isolated from aborted bovine fetuses indicating that it has clinical relevance in cattle. Cff is associated with sporadic cases of bovine abortion whereas Cfv is associated with endemic abortion and fertility problems in certain areas.

Although *C. fetus* is primarily recognised as a pathogen in animals, Cff is occasionally diagnosed as an opportunistic pathogen in humans. Infections usually occur in pregnant or immuno-compromised individuals and are often systemic with a variety of complications depending on the site of infection (Wagenaar *et al.*, 2014). Laboratory manipulations should be performed 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*).

2. Taxonomy

Campylobacter fetus is one of the 33–68 currently recognised species within the genus *Campylobacter* (<http://www.bacterio.net/campylobacter.html> <https://lpsn.dsmz.de/search?word=campylobacter>). Three subspecies of *C. fetus* have been recognised: *C. fetus* subspecies *testudinum* (Cft), *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis*, of which Cft is reptile-associated and both Cff and Cfv are mammal-associated. Cfv includes a variant designated Cfv biovar *intermedius* (Florent *et al.*, 1959; Veron & Chatelain, 1973). Although the clinical signs related to infections by Cfv and Cff overlap, they were originally defined by the differences in clinical presentation (Florent *et al.*, 1959; Veron & Chatelain, 1973).

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods^(a) available for diagnosis of bovine genital campylobacteriosis 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^(b)						
Culture (including subspecies identification)	☑☑☑	☑☑☑	☑☑☑	☑☑☑	☑☑☑	□□□
IFAT	☑☑□	☑☑□	☑☑□	☑☑□	☑☑□	□□□
MAB-based capture ELISA	☑☑□	☑☑□	☑☑□	☑☑□	☑☑□	□□□
Detection of immune response						
Antibody ELISA	□□□	□□□	□□□	□□□	□□□	☑□□

Key: ☑☑☑ = recommended for this purpose; ☑☑□ = recommended but has limitations;

☑□□ = suitable in very limited circumstances; □□□ = not appropriate for this purpose.

IFAT = immunofluorescent antibody test; MAB = monoclonal antibody; ELISA = enzyme-linked immunosorbent assay.

^(a)Culture is the gold standard, but there are no data on specificity and sensitivity at the herd level.

The characteristics of the additional tests described (e.g. IFAT and MAB-based capture ELISA) are compared with culture at the sample level, not at the herd level.

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

Culturing and identification of the organism is a suitable test for certifying individual animal prior to movement. The validated sensitive and specific monoclonal antibody (MAb)-based capture enzyme-linked immunosorbent assay (ELISA) screening procedure (Brooks *et al.*, 2004; Devenish *et al.*, 2005) can accurately detect *C. fetus* subsp. from incubated Clarke's transport enrichment medium (TEM). Only positive ELISA results are cultured for confirmation of the specific subspecies using standard isolation and identification methods. This procedure has the advantage of more rapid turnaround times for reporting, and ease, compared with traditional cultural methods.

Cfv and Cff can be differentiated by the 1% glycine tolerance test and H₂S production in cysteine-rich medium; Cfv is not able to grow in the presence of 1% glycine and not able to produce H₂S from cysteine-rich medium, whereas Cff is 1% glycine tolerant and H₂S production positive (Florent *et al.*, 1959; Veron & Chatelain, 1973). Neither DNA–DNA hybridisation (Harvey & Greenwood, 1983) nor protein-banding patterns using polyacrylamide gel electrophoresis (PAGE) of whole cell proteins (Vandamme *et al.*, 1990) revealed any major difference between Cfv and Cff. MALDI-TOF (matrix assisted laser desorption ionisation–time of flight) mass spectrometry can be used to identify *C. fetus* at species level (Bessede *et al.*, 2011), but this assay is not able to differentiate between Cff and Cfv. Of all described molecular tests, only polymerase chain reaction (PCR) assays targeting the *nahE* gene, both conventional and real-time (Abril *et al.*, 2007; van der Graaf-van Bloois *et al.*, 2013), and multilocus sequence typing (MLST) and amplified fragment length polymorphism (AFLP) (van Bergen *et al.*, 2005a; Wagenaar *et al.*, 2001) are able to identify *C. fetus* reliably at the species level. Several molecular methods have been described claiming to differentiate between Cff and Cfv, including PCR (Abril *et al.*, 2007; Hum *et al.*, 1997; Tu *et al.*, 2005; van Bergen *et al.*, 2005c; van der Graaf-van Bloois *et al.*, 2024; Wang *et al.*, 2002), pulsed-field gel electrophoresis (PFGE) (On & Harrington, 2004), MLST (van Bergen *et al.*, 2005a) and AFLP (Wagenaar *et al.*, 2001) (see also Section B.1.9), but none of these molecular tests of which only the PCR targeting the *asd* gene is able to identify the *C. fetus* isolates reliably to subspecies level (Iraola *et al.*, 2015; van der Graaf-van Bloois *et al.*, 2013; 2024). Whole genome sequencing (WGS) can be used to differentiate the mammal-associated for reliable genomic differentiation of *C. fetus* strains based on their core genomes (van der Graaf-van Bloois *et al.*, 2014), but this method is not in full concordance with the phenotypic subspecies identification of *C. fetus* strains.

1. Detection and identification Isolation and detection of the agent

1.1. Collection of specimens

1.1.1. Male: preputial smegma and semen

In bulls, smegma may be obtained by different methods (da Silva Silveira *et al.*, 2018): scraping (Tedesco *et al.*, 1977), aspiration (Campero *et al.*, 2003), and washing (Clarke & Dufty, 1978). Note that the same sample, collected using the same sampling method, can be used for the detection of *Trichomonas foetus* (Waldner *et al.*, 2017) (see Chapter 3.4.14 *Trichomonosis*).

Smegma can be used for isolation of the organism or is rinsed into a tube with approximately 5 ml phosphate buffered saline (PBS, pH 7–7.3, concentration 1×, components: 138 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄·7H₂O, in 1.5 mM KH₂PO₄) with 1% formalin for diagnosis by immunofluorescence. Smegma can also be collected from the artificial vagina after semen collection, by washing the artificial vagina with 20–30 ml of PBS.

For preputial washing, 20–30 ml of PBS is introduced into the preputial sac. After vigorous massage for 15–20 seconds, the infused liquid is collected.

Semen is collected under conditions that are as aseptic as possible. Semen samples must be diluted with PBS and are inoculated directly on to culture medium or into transport and enrichment medium.

1.1.2. Female: cervico vaginal mucus

Samples may be obtained by aspiration or washing the vaginal cavity. Hollow plastic scraping tools can also be used. The samples can also be used for *Trichomonas foetus* detection (see chapter 3.4.14)

For aspiration, the vulva region is cleaned, and an artificial insemination (AI) pipette or Cassou pipette (blue sheath type) is inserted into the vaginal cavity so that the anterior reaches the cervix (Campero *et al.*, 2003). Gentle suction is applied while moving the pipette gently backwards and forwards. The pipette is removed, and the collected mucus is inoculated directly on to culture medium or into transport and enrichment medium.

Cervico-vaginal mucus (CVM) may also be collected by washing the vaginal cavity: 20–30 ml of PBS is infused into the cavity through a syringe attached to an AI pipette. The fluid is sucked out and re-infused four to five times before being collected and spread directly on to culture medium

or added to transport and enrichment medium. Washing fluid in the vaginal cavity may also be collected by a tampon or gauze held inside the vagina for 5–10 minutes after PBS infusion. Samples of CVM obtained by suction may be diluted with PBS or sown directly on to culture medium or transport and enrichment medium.

CVM is transferred into approximately 5 ml of PBS with 1% of formalin for immunofluorescence testing.

1.1.3. Aborted fetuses, placentas

The placenta as well as the liver, lungs and stomach contents of the fetus provide the best samples for isolation of the causative bacteria. Samples are collected aseptically and inoculated into transport and enrichment medium, or into PBS with 1% formalin for IFA testing.

1.2. Transport of specimens

The use of a transport medium is essential if the specimens are not processed in the laboratory within the same day after collection. For dispatch to the laboratory, specimens must be placed in an insulated container (within the temperature range 4–8°C) protected from light and shipped to reach the laboratory as soon as possible, preferably on the same day as sampling.

Various transport and enrichment media are available, such as Clark's, Lander's, SBL, Foley's and Clark's, Weybridge's, Cary-Blair's (Garcia *et al.*, 1984; Hum *et al.*, 1994; Monke *et al.*, 2002), Thomann's transport and enrichment (TTE) medium (Harwood *et al.*, 2009).

Some of the transport and enrichment media mentioned above contain cycloheximide. Because of its potential toxicity, amphotericin B can be used as an alternative.

1.3. Treatment of specimens

On arrival at the laboratory, specimens should be inoculated directly on to culture medium, or, if required immediately, processed further.

1.3.1. Genital tract samples

The total volume of the preputia washings may be centrifuged (3500 *g*) to concentrate the sample. The final sample (reduced to 500 µl) may be inoculated on to the culture medium (directly on Skirrow agar plate, using the filter method, and to TTE medium (Harwood *et al.*, 2009).

If the CVM is not very viscous it can be inoculated directly or diluted with an equal volume of PBS (pH 7–7.3, concentration 1×, components: 138 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄·7H₂O, in 1.5 mM KH₂PO₄). When the CVM is very viscous, it may be necessary to liquefy it by adding an equal volume of L-cysteine solution (100 mM *N*-Acetyl-L-cysteine, CAS number 616-91-1³⁴, dissolved in PBS and sterilised by membrane filtration). After 15–20 minutes, the diluted and liquefied mucus can then be inoculated on to isolation medium.

For the filter method, sterile cellulose acetate membranes of 0.65 µm pore size are placed on the surface of non-selective agar supplemented with 5% blood. A suspension of the sample is made in in PBS or saline, and 10–15 drops of this suspension are placed on top of the membrane and allowed to filter passively at 37°C (microaerobic conditions are not required). After 30–40 minutes, the filter membranes are removed and the culture plates incubated for 4–2–2–3 days at 37°C under microaerobic conditions (Steele & McDermott, 1984).

1.3.2. Aborted fetuses, placentas

Fetal stomach contents are inoculated directly on to culture medium. Internal organs or pieces of organs are flamed to disinfect the surface and are subsequently homogenised. The homogenate is inoculated on to culture medium.

After washing placental membranes with PBS to eliminate the majority of the surface contamination, the chorionic villi are scraped and the scrapings are transferred to culture medium.

34 This is the unique number for this chemical assigned by Chemical Abstracts Service (CAS).

1.4. Detection and identification ~~Isolation~~ of *Campylobacter fetus*

1.4.1. Culture media for isolation

Many media are currently in use for the bacteriological diagnosis of BGC. It should be noted that several media used for the isolation of *Campylobacter* spp. are not suitable for the isolation of *C. fetus* because of antimicrobials (e.g. cephalosporins) that may inhibit *C. fetus* growth (van Bergen *et al.*, 2005b; van der Graaf-van Bloois *et al.*, 2023). Most culture media contain cycloheximide. Because of its potential toxicity, this antifungal agent can be replaced by amphotericin B. The recommended selective medium for isolation of *C. fetus* is TTE (Harwood *et al.*, 2009). This is based on Skirrow's medium, but with the selective supplements reduced to 75% of the original recommendations, i.e.: polymyxin B sulphate (1.9 IU/ml), trimethoprim (3.8 µg/ml), vancomycin (7.5 µg/ml) and 5 mg/litre amphotericin B. After 48 hours of enrichment, the enrichment broth can be plated on either Skirrow's selective plates or non-selective plates using the filtration method. Skirrow's selective plates are a blood-based agar containing 5–7% (lysed) defibrinated blood and Skirrow selective supplement.

Alternatively, a non-selective blood-based (5–7% blood) medium in combination with filtration (0.65 µm) can be used; however, it may be less sensitive when compared with a selective medium.

Quality control of each batch of media should be performed using control strains.

1.4.2. Incubation conditions

Plates or tubes with TTE medium are incubated at 37°C and under microaerobic atmosphere of 5–10% oxygen, 5–10% carbon dioxide and preferably 5–9% hydrogen for optimal growth (Vandamme, 2000). Appropriate microaerobic conditions may be produced by a variety of methods. In some laboratories the suitable atmosphere is created by a gas replacement in a jar. Gas generator kits are also available from commercial sources. Variable atmosphere incubators can also be used.

Conditions of culture and incubation are systematically verified by using control strains of *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis*. Such controls should be set up for each isolation attempt.

1.5. Detection and identification ~~Identification~~ of *Campylobacter* species

1.5.1. Colony morphology

Colonies of *C. fetus* usually appear on culture media after 2–5 days. To prevent overgrowth of specific colonies by contaminants, it is recommended that the media be evaluated daily and suspicious colonies be subcultured. After 3–5 days of incubation, colonies measure 1–3 mm in diameter. They are slightly grey-pink, round, convex, smooth and shiny, with a regular edge.

1.5.2. Macroscopic morphology

Campylobacter is motile, a property that may disappear during sub-culturing. *Campylobacter* often takes the form of a thin, curved bacillus, 0.3–0.4 µm wide and 0.5–8.0 µm long. Short forms (comma-shaped), medium forms (S-shaped/flying seagull-shaped), and long forms (helical with several spirals) may be observed simultaneously in the living state. Old cultures may contain coccoid bacteria.

1.5.3. Biochemical tests

See Table 2.

1.5.4. Atmosphere

Campylobacter does not grow under aerobic conditions.

1.6. Detection of *Campylobacter fetus* by the immunofluorescence method

The immunofluorescence antibody test (IFAT) can be applied to detect the organism directly from samples or to confirm the identification of a strain after isolation. This test was able to detect *C. fetus* in contaminated field samples. The detection limit of this test is 10⁴ and 10² colony-forming units/ml in non-

centrifuged and centrifuged preputial washings, respectively (da Silva Silveira *et al.*, 2018). IFAT cannot differentiate between different subspecies.

1.6.1. Preparation of immune sera

Campylobacter strains, preferably standard strains from recognised culture collections (*C. fetus* subsp. *venerealis* or *C. fetus* subsp. *fetus*), are grown on blood-based medium at 37°C under microaerobic conditions for 3 days. The organisms are harvested into PBS, and washed twice by centrifugation. Rabbits aged 3 months are inoculated intramuscularly with 2 ml of 10¹¹ organisms/ml of a *C. fetus* subspecies resuspended in PBS and Freund's incomplete adjuvant. Inocula are administered at four sites, 0.5 ml at each site. The animals are bled before inoculation and at weekly intervals thereafter. When the serum titres reach high levels, as estimated by the immunofluorescence test or agglutination test, 0.1–1.0 ml of 10¹⁰ viable organisms/ml are injected intravenously. The rabbits are bled for serum 7 days later. The rabbit sera are pooled. A conjugate prepared from chicken IgY was described as an alternative to rabbit antibodies. MAbs that can be used for immunodiagnostic detection of *C. fetus* have been described (Brooks *et al.*, 2002).

1.6.2. Preparation of conjugates

Conjugates are prepared as described by Harlow & Lane (1988). The working dilution of the conjugate is determined by checkerboard titration against smears of a *C. fetus* culture using positive and negative control dilutions and selecting twice the lowest concentration that produces brilliant fluorescence with *C. fetus* bacteria.

1.6.3. Sample preparation

The genital fluid (fetal abomasal content, preputial smegma or CVM) samples are rinsed into approximately 5 ml PBS + 1% formalin. Two centrifugation steps are carried out. First, samples are centrifuged at 600 *g* for 10 minutes at 4°C to remove debris. Subsequently, the supernatant is centrifuged at 8000 *g* for 30 minutes at 4°C. The pellet is dissolved in ~100 µl remaining supernatant.

1.6.4. Immunofluorescence test (Mellick *et al.*, 1965)

The sample (20 µl) is applied in duplicate to microscope slides. The material is air-dried and fixed in acetone at –20°C for 30 minutes or ethanol at 18–25°C for 30 minutes. Glass slides will be air-dried and the fluorescein isothiocyanate isomer (FITC)-conjugated antiserum is added at the appropriate dilution. Staining is carried out in a humid chamber at 37°C for 30 minutes in dark condition. The slides are then washed three times for 10 minutes in PBS. The slides are mounted in buffered glycerol (90% glycerol: 10% PBS). The cover-slips are sealed to prevent drying, and the slides are examined under ultraviolet light in a fluorescent microscope. Positive and negative control slides will be used each time the test is done. *Campylobacter fetus* subsp. *venerealis* and *C. fetus* subsp. *fetus* reference strains are used as positive controls, and another *Campylobacter* species is used as negative control. Samples that show fluorescent bacteria presenting the typical morphology of *C. fetus* are considered positive.

1.7. Biochemical identification of *Campylobacter fetus* subspecies

Tests described in Table 2 must be done on pure cultures.

Table 2. Differential characteristics of *Campylobacter* species potentially isolated from the bovine genital tract and aborted fetuses (according to Bergey's Manual 2nd edition, 2005)

	25°C	42°C	Oxidase	Catalase	NaCl 3.5%	Glycine 1%	H ₂ S cysteine
<i>C. fetus</i> subsp. <i>venerealis</i>	✓	✓ ^(a)	+	V	–	–	–
<i>C. fetus</i> subsp. <i>fetus</i>	✓	✓ ^(a)	+	+	–	+	+
<i>C. jejuni</i>	–	✓ ^(b)	±	✓ ^(c)	–	✓	±
<i>C. hyointestinalis</i>	–	±	+	+	–	V	n.d.
<i>C. sputorum</i>	–	±	+	V	+	+	n.d.

^(a)Although *C. fetus* does not belong to the thermophilic *Campylobacters*, a considerable number of strains of this species grows at 42°C;
^(b)*C. jejuni* subsp. *jejuni* is positive, *C. jejuni* subsp. *doylei* is negative;
^(c)*C. jejuni* subsp. *jejuni* is positive, *C. jejuni* subsp. *doylei* is variable;
+ = positive reaction or growth and – = negative reaction or absence of growth of the strain on an appropriate medium under specified conditions (see Section B.1.4); V = variable results; n.d. = not determined.

~~1.7.1. Growth at 25°C and 42°C~~

~~A cell-suspension (~McFarland No. 1) is inoculated on to two blood-based medium plates. Each plate is incubated under the specified atmospheric conditions (see Section B.1.4.2) at 25°C and 42°C. Control strains are tested in parallel.~~

1.7.2-1. Oxidase and catalase

Tests are performed according to a standard bacteriological protocol. Control strains are tested in parallel.

1.7.3-2. Growth in the presence of sodium chloride

A cell-suspension is inoculated on to blood medium containing 3.5% NaCl (15 ml of blood medium + 2.04 ml of 5 M sodium chloride solution), and on to plain blood medium. Incubation is performed under the specified atmospheric conditions (see Section B.1.4.2). Control strains are tested in parallel.

1.7.4-3. Growth in the presence of 1% glycine

A cell-suspension (~McFarland No. 1) is inoculated on to a glycine medium (15 ml of blood-based medium + 1.65 ml of 10% aqueous solution of filter sterilised glycine), and on to the same medium without glycine. Incubation is performed under the specified atmospheric conditions (see Section B.1.4.2). Two control strains (Cff and Cfv) are tested in parallel. As all strains are fastidious, small changes in media can be important, and lack of growth in the presence of glycine should be considered to be a presumptive test for *C. fetus* subsp. *venerealis*. The reproducibility of the assay is poor and intermediate strains have been described (Salama *et al.*, 1992; Van Bergen *et al.*, 2005a). Furthermore, the reliability of the 1% glycine tolerance test can be influenced by the fact that glycine tolerance can be transduced by phages (Chang & Ogg, 1971).

1.7.5-4. Hydrogen sulphide production in cysteine medium

The H₂S test is done in a *Brucella* broth medium containing 0.02% cysteine. A cell suspension (~McFarland No. 1) is inoculated into the medium. H₂S production is detected by a lead acetate strip that is attached inside the top of the tube for 72 hours. Blackening of the lead acetate strip is considered as a positive reaction. Control strains are tested in parallel.

1.8. Monoclonal antibody-based capture ELISA

The MAb-based capture ELISA procedure can be used to detect the presence of *C. fetus* species cultured in Clarke's TEM. The validated procedure and reagents are described by Brooks *et al.* (2004) and Devenish *et al.* (2005). Briefly, the samples (preputial washes, vaginal mucus, fetal fluids, placental tissues, liver tissue) are placed in Clarke's TEM and incubated for 4–5 days. Approximately 1.5 ml of the TEM fluids is withdrawn, heated and tested by ELISA. A rabbit polyclonal antiserum (to six different *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* serotype A and B strains) is used to capture antigen from the TEM fluid. The detection of any captured antigen is accomplished with further testing using four mouse MAbs specific to lipopolysaccharide (LPS) epitopes; *C. fetus* subsp. core LPS (1 MAb: M1825), serotype A-specific side chain LPS (2 MAbs: M1177 and M1194) and serotype B-specific side chain LPS (1 MAb: M1183). The test has been shown to be 100% sensitive and 99.5% specific for the detection of *C. fetus* species from the TEM fluids, and large numbers of samples can be tested at the same time.

1.8.1. Test procedure

- i) **Solid phase:** ELISA plate wells are coated with an optimal dilution of a rabbit polyclonal anti-*C. fetus* subsp. serum (in 0.06 M carbonate buffer, pH 9.6) and incubated for 18 hours at room temperature (RT). Coated ELISA plates can be sealed and stored at –20°C for 1 month. Prior to testing, ELISA plates, at RT, are washed four times using 0.01 M PBS containing 0.15 M NaCl and 0.05% Tween 20 (PBST).
- ii) **Controls:** There are three control antigens: 1) *C. fetus* subsp. *fetus* (ATCC 27374), a serotype B strong positive control that binds with the LPS core MAb (M1825) and the single serotype B specific MAb (M1183); 2) *C. fetus* subsp. *venerealis* (ATCC 19438), a weak serotype A positive control that binds to the LPS core MAb (M1825) and the two serotype

A-specific MAbs (M1177 and M1194); and 3) *C. sputorum* biovar *sputorum*, a negative control that does not bind with any of the four MAbs. Lots of the three control antigens are grown, washed, concentrated and stored at -20°C .

- iii) **Testing samples:** After 4–5 days' incubation at 37°C of inoculated TEM samples, approximately 1.5 ml of fluid is withdrawn from the TEM vials. Optimally diluted control antigens and undiluted TEM fluid samples are heated at 100°C for 15 minutes and cooled to RT. Each heated test fluid sample is added in duplicate and each heated control antigen is inoculated in quadruple to appropriate wells in the solid-phase ELISA plate and incubated for 1 hour at RT.
- iv) **Detector:** To economise on reagents, screening of fluids is done initially with the core MAb M1825. Diluted MAb M1825 (optimally in PBST) is added to each well of the ELISA plates and the plates are incubated for 1 hour at RT. Fluids that are positive using MAb M1825 are further tested using all four MAbs.
- v) **Conjugate:** Horseradish-peroxidase goat anti-mouse immunoglobulin G (heavy and light chains) conjugate, optimally diluted in PBST, is added to all wells and plates are incubated for 1 hour at RT.
- vi) **Substrate:** 3,3',5,5'-tetramethylbenzidine-hydrogen peroxide substrate is added to wells and the plates are placed on an orbital shaker for 10 minutes at RT. Optical densities (OD) are immediately measured at 620 nm (OD_{620}) using an ELISA reader.

All reagent lots, including capture antiserum, control antigens, mouse MAbs and conjugate are tested beforehand by checkerboard titration to determine optimal dilutions used in routine testing of sample fluids. Throughout the test procedure, 100 μl volumes are used and plates are washed four times between stages using PBST.

- vii) **Interpretation of the results:** To be ELISA positive, the fluid must be positive against not only the core MAb but also to at least one of the serotype-specific MAbs. In initial screening using MAb M1825, the mean OD_{620} of the each test sample is divided by the mean OD_{620} obtained from strong positive *C. fetus* subsp. *fetus* serotype B control antigen and multiplied by 100% to obtain a per cent positivity (%P). Any test fluid with a %P greater than or equal to 14% is considered positive and tested further with all four MAbs in the procedure. A repeat positive with M1825 and an OD_{620} value greater than or equal to 0.2 with at least one of the other three serotype-specific MAb is a positive ELISA result for the detection of *C. fetus* ssp. in the original test fluid sample. All TEM vials corresponding to ELISA positive fluid samples are cultured as described in Section B.1.4 *Isolation of Campylobacter fetus* for confirmation and to determine the subspecies of the cultured isolate.

1.9. Molecular identification of *Campylobacter fetus* subspecies

The use of clinical samples for direct PCR has not been successfully demonstrated and can result in nonspecific amplification. The method is therefore not recommended for primary diagnosis. It is however possible to perform PCR from TTE medium after enrichment.

Several molecular methods for the identification of *C. fetus* subspecies have been described, including 16S sequencing (Gorkiewicz *et al.*, 2003; On & Harrington, 2004), PFGE (On & Harrington, 2004), AFLP (Wagenaar *et al.*, 2004), and MLST (van Bergen *et al.*, 2005a). MLST was recommended for the differentiation of Cff and Cfv strains; a recent study however, a study described a Cff strain that was isolated with the Cfv-associated MLST ST-4 genotype (Iraola *et al.*, 2015), showing that MLST is also not fully reliable for *C. fetus* subspecies differentiation. ~~Whole genome sequencing-WGS can be used for a very reliable characterisation genomic identification of *C. fetus* strains (van der Graaf-van Bloois *et al.*, 2014-2024), but this method is expensive and currently not widely used in diagnostic laboratories. Furthermore, the web-page <https://klif.uu.nl/cfvcatch> was developed as an online tool for identification of *C. fetus* genomes to the subspecies level, to perform phylogenetic analysis with reference genomes and to identify antimicrobial resistant genes.~~

~~PCR assays are the best option for routine diagnostic laboratories would be served best by a PCR assay. Several PCRs have been claimed to be subspecies specific, including those developed by Hum *et al.* (1997), Wang *et al.* (2002), Tu *et al.* (2005), van Bergen *et al.* (2005c), and Abril *et al.* (2007) and van der Graaf-van Bloois *et al.* (2024). However, a only the recently described PCR assay that identifies targeting the *asd* gene can identify *C. fetus* isolates reliably to the subspecies level is not available (van der Graaf-van Bloois *et al.*, 2013-2024) (Table 3).~~

Conventional PCR

Pathogen/ target gene	Primer (5'–3')	Concentration	Cycling parameters ^(a)
Method: Abril et al. (2007), amplicon size: 390 bp			
NahE	CFETSpp-L: GGT-TAT-TTT-TTA-TAA-CTG-TAG-GAA-TGC-AGA-T CFETSpp-R: GAT-CGC-TTA-AAT-CTT-GTA-CTT-TTA-GCT-TTT	20 nM	35 cycles of: 95°C/30 sec 48°C/40 sec / 72°C 30 sec
Method: Van Der Graaf-Van Bloois et al. (2024), amplicon size: Cff: 335 bp, Cfv 329 bp			
Asd_Cff	ASD_forw: CAC-TTG-TGT-TCG-TGT-TC ASD_Cff_rev: TTT-TTA-GCT-AAT-GGT-TTA-ATT-C	10 nM	35 cycles of: 95°C/20 sec 58°C/20 sec and 72°C 30 sec
Asd_Cff	ASD_forw: CAC-TTG-TGT-TCG-TGT-TC ASD_Cfv_rev: TTT-TTA-GCT-AAT-GGT-CTT-G	10 nM	35 cycles of: 95°C/20 sec 58°C/20 sec and 72°C 30 sec

^(a)A denaturation step prior to cycling has not been included.

PCR fragments should be visualised on a 2% agarose gel stained with for example Midori Green Advanced DNA staining (GC tech) and a positive result is indicated by visible DNA fragments with the sizes indicated in the table above.

Real-time PCR

Pathogen/ target gene	Primer (5'–3')	Concentration	Cycling parameters ^(a)
Method: Method: Van Der Graaf-Van Bloois et al. (2013), amplicon size: Cff: 335 bp, Cfv 329 bp			
NahE	nahE_F: TGT-TAT-GGT-GAT-CAA-AAT-AGC-TGT-TG nahE_R: GAG-CTG-TTT-TTA-TGG-CTA-CTC-TTT-TTT-TA nahE_P: FAM-TGT-ATA-TGC-ACT-TTT-AGC-AAC-TT-NFQ	nahE_F: 12 nM nahE_R: 12 nM nahE_P: 4 nM	40 cycles of: 95°C/3 sec and 60°C/30 sec
Method: Van Der Graaf-Van Bloois et al. (2024)			
asd	ASD_RT_forw: TCT-TTG-GTG-CGT-TGC-TGA-TCA ASD_RT_rev: GAT-GAC-GGG-CAA-AAT-AGT-CAC-GT ASD_Cff_P: FAM-TGG-TGT-CAA-ATA-CAA-GAA-TTA-AAC-C-BHQ ASD_Cfv_P: HEX-AAA-TGG-TGT-CAA-ATA-CAA-GAC-CA-BHQ	ASD_RT_forw: 12 nM ASD_RT_rev: 12 nM ASD_Cff_P: 4 nM ASD_Cfv_P: 4 nM	45 cycles of: 95°C/10 sec and 58°C/30 sec

^(a)A denaturation step prior to cycling has not been included.

Table 3. Sensitivity and specificity of *C. fetus* (sub)species identification of PCR assays

PCR assay	Identification	Target	Sensitivity ^(a)	Specificity ^a
Abril et al., 2007	<i>C. fetus</i> Cfv	nahE ISCfe1	100% (143/143) 97% (58/60)	100% (12/12) 100% (95/95)
Van Bergen et al., 2005c	Cfv	unknown	45% (27/60)	100% (95/95)
Hum et al., 1997	<i>C. fetus</i> Cfv	cstA parA	100% (143/143) 58% (46/70)	100% (12/12) 83% (79/95)
McMillen et al., 2006	Cfv	parA	53% (32/60)	100% (95/95)
Wang et al., 2002	Cff	sapB2	76% (63/83)	72% (52/72)
Van der Graaf-van Bloois et al., 2013	<i>C. fetus</i>	nahE	100% (143/143)	100% (12/12)
Van der Graaf-van Bloois et al., 2024	Cff Cfv	Asd	Cff: 100% (104/104) Cfv: 100% (38/38)	Non <i>C. fetus</i> 100% (38/38)

^(a)Represented as percentage (number of correct identified strains/total number of strains). Study included: 143 *C. fetus* strains, 60 Cfv strains, 83 Cff strains, 12 non-*fetus* *Campylobacter* strains, 95 non-Cfv strains and 72 non-Cff strains (van der Graaf-van Bloois et al., 2013).

The recently described PCR assay targeting the *asd* gene identifies both subspecies (Cff and Cfv) reliably van der Graaf-van Bloois *et al.*, 2024). The *asd* PCR can be assessed *in silico* on sequenced genomes using a web interface containing the Cfvcatch tool (<https://klif.uu.nl/cfvcatch>).

The multiplex PCR assay described by Abril *et al.* (2007) proved to be reliable for the correct identification of *C. fetus* species, with 100% sensitivity and 100% specificity, though the Cfv-specific target (IS-element) described for this PCR is 97% sensitive and cannot be used for a reliable differentiation of Cff and Cfv isolates (van der Graaf-van Bloois *et al.*, 2013). The *C. fetus* target of this PCR, gene *nahE*, can be used to identify *C. fetus* species and has also been developed as a real-time assay (McGoldrick *et al.*, 2013; van der Graaf-van Bloois *et al.*, 2013).

The PCR described by Van Bergen *et al.* (2005c) is able to detect Cfv strains as defined by AFLP, but the assay does not identify the *C. fetus* subsp. *venerealis* biovar *intermedius*. Therefore, this PCR is not suitable for diagnostic purposes (van der Graaf-van Bloois *et al.*, 2013).

The multiplex PCR described by Hum *et al.* (1997) enables the amplification of a *C. fetus*-specific DNA fragment (approximately 200 bp smaller than the 960 bp described in the original publication), as well as a *C. fetus* subsp. *venerealis*-specific fragment. Comparison of this PCR against AFLP and MLST (van Bergen *et al.*, 2005a) and against the glycine test (Willoughby *et al.*, 2005) confirms that PCR can give false positive and negative reactions (van der Graaf-van Bloois *et al.*, 2013). In a recent study, this PCR showed a positive result with a *C. hyointestinalis* strain isolated from a bull (Spence *et al.*, 2011). This observation renders the Cfv-specific target gene *parA*, and consequently all other PCR assays using this target, unsuitable for diagnostic purposes.

The PCR described by Wang *et al.* (2002) reveals only a *C. fetus* subsp. *fetus*-specific product. These results were obtained only for a very limited number of strains. Evaluations of its value for subspecies differentiation using larger sets of strains yielded both false positive and negative reactions (Van Bergen *et al.*, 2005c; Van der Graaf-van Bloois *et al.*, 2013).

The random amplification of polymorphic DNA (RAPD) PCRs were described by Tu *et al.* (2005) but apparently have been evaluated with a very limited number of *C. fetus* subsp. *venerealis* strains. In a recent study, the sensitivity of this assay was shown to be very low (Van der Graaf-van Bloois *et al.*, 2013).

2. Serological tests – antibody detection

An ELISA is available for the detection of antigen-specific secretory IgA antibodies in the vaginal mucus following abortion due to *C. fetus* subsp. *venerealis*. These antibodies are long lasting, and their concentration remains constant in the vaginal mucus for several months (Hum *et al.*, 1991). This assay has been used to screen for BGC in cattle herds with infertility and abortions; the specificity was found to be 98.5%. The sensitivity could not be estimated (da Silva Silveira *et al.*, 2018) so it is not yet considered as validated. The ELISA does not differentiate an antibody response to the different subspecies.

Initial sampling can be done after the early involution period (usually 1 week after abortion) when mucus becomes clear.

2.1. Antigen preparation and coating

Cultures are transferred to PBS with 0.5% formalin for 1 hour, centrifuged at 17,000 *g*, washed twice with PBS, and then resuspended in 0.05 M carbonate buffer, pH 9.6. The final absorbance is adjusted to OD_{610 nm} = 0.21. Flat-bottomed polystyrene microtitre plates coated with 10 µl of antigen are left overnight at 4°C, and then stored at –20°C. Before use, the plates are rinsed twice with distilled water and then tapped gently to remove moisture.

2.1.1. Test procedure

- i) Diluted vaginal mucus (100 µl) is added to each well, and the plate is incubated at 37°C for 2 hours. The plates are then washed as before, and 100 µl of rabbit anti-bovine IgA is added. After 2 hours incubation at 37°C, the plates are washed and 100 µl of goat anti-rabbit IgG conjugated to horseradish peroxidase is added to each well. After a further 2 hours incubation at 37°C, the plates are washed, and 100 µl of substrate is added (0.8 mg/µl 5 amino-salicylic acid; pH 6.0), immediately activated by the addition of 2% 1 M hydrogen peroxide. The plates are left at room temperature for 30 minutes and the reaction is stopped by the addition of 50 µl of 3 M sodium hydroxide. The absorbance is measured on an ELISA reader at 450 nm. Each sample is tested in duplicate, and positive and negative controls are

included in each plate. The absorbance measurements yielded by the test sample are corrected for the absorbance measurement of positive and negative controls according to the formula:

$$\text{Result} = \frac{\text{Absorbance}_{\text{sample}} - \text{Absorbance}_{\text{negative control}}}{\text{Absorbance}_{\text{positive control}} - \text{Absorbance}_{\text{negative control}}} \times 100$$

- ii) The test is considered to be positive if the result is above 40. Vaccinated animals will not react to IgA ELISA as their vaginal mucus contains only IgG isotype antibodies.

An ELISA for the detection of the serum humoral IgG response after vaccination has also been described (Cobo *et al.*, 2004).

C. REQUIREMENTS FOR VACCINES

There are several commercial vaccines available around the world, however vaccine efficacies have either not been reported for many decades or have not been reported favourably (Cobo *et al.*, 2003). Certain jurisdictions advocate the use of these vaccines, however WOA is not currently in a position to recommend an international standard for such products.

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NB: There is a WOA Reference Laboratory for campylobacteriosis (please consult the WOA Web site: <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>).

Please contact the WOA Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for bovine genital campylobacteriosis

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2021.

MEETING OF THE BIOLOGICAL STANDARDS COMMISSION

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CHAPTER 3.4.10.

**HAEMORRHAGIC SEPTICAEMIA
(*PASTEURELLA MULTOCIDA*
SEROTYPES 6:B AND 6:E)**

SUMMARY

Haemorrhagic septicaemia (HS) is a major disease of cattle and buffaloes characterised by an acute, highly fatal septicaemia with high morbidity and mortality. It is caused by certain serotypes of Pasteurella multocida that are geographically restricted to some areas of Asia, Africa, the Middle East and southern Europe.

The diagnosis of HS depends on the isolation of the causative organism, P. multocida, generally from the blood or bone marrow of a dead animal, by cultural and biological methods, and the identification of the organism by biochemical, serological and molecular methods.

Isolation and detection of the agent: *Pure cultures of P. multocida can be obtained by streaking materials on to artificial media and the subsequent identification on the basis of the morphological, cultural, and biochemical characteristics of P. multocida.*

Conventionally, the identification of the specific serotype is carried out using one or more serological methods. These include rapid slide agglutination, indirect haemagglutination for ‘capsular’ typing using sheep red blood cells coated with bacterial extracts, ‘somatic’ typing by agar gel immunodiffusion tests using heat-treated cell extracts, or agglutination using acid-treated cells. Confirmation of the isolates can be made using molecular techniques.

Serology: *Serological tests for detecting specific antibodies are not normally used for diagnostic purposes.*

Requirements for vaccines: *Effective vaccines against HS are formalin-killed bacterins, or dense bacterins with adjuvants. The latter enhance the level and prolong the duration of immunity.*

Seed cultures for the production of vaccines should contain capsulated organisms. Vaccines are standardised as to their bacterial density on the basis of turbidity tests and dry bacterial weight. Potency tests are carried out in mice and/or rabbits.

A. INTRODUCTION

Haemorrhagic septicaemia (HS) is a major disease of cattle and buffaloes occurring as catastrophic epizootics in many Asian and African countries, resulting in high mortality and morbidity (Bain *et al.*, 1982; Carter & De Alwis, 1989; Chanda *et al.*, 2024; De Alwis, 1992; Mustafa *et al.*, 1978; Shivachandra *et al.*, 2011; Singh *et al.*, 1996). The disease has been recorded in wild mammals in several Asian and European countries (Carigan *et al.*, 1994; Rosen, 1981). In many Asian countries disease outbreaks mostly occur during the climatic conditions typical of monsoon (high humidity and high temperatures). The disease is caused by *Pasteurella multocida*, a Gram-negative

coccobacillus residing mostly as a commensal in the nasopharynx of animals. The Asian serotype B:2 and the African serotype E:2 (Carter and Heddlestone system), corresponding to 6:B and 6:E (Namioka-Carter system), are mainly responsible for the disease. In wild ruminants, serotype B:2,5 is predominantly present while serotype B:3,4 also has been reported from fallow deer (Aalbak *et al.*, 2009). The association of other serotypes, namely A:1, A:3 with a HS-like condition in cattle and buffaloes in India has been recorded (Kumar *et al.*, 1996–2004). The geographical distribution of HS includes some areas of Asia, Africa, the Middle East and southern Europe. It has never been confirmed in Mexico, Central or South America.

Clinically, HS caused by B:2 or E:2 strains is typified by fever, respiratory distress with nasal discharge, and frothing from the mouth, leading eventually to recumbency and death. Infection with serotypes A:1 and A:3 predominantly involves pneumonia and death. Septicaemia is the main characteristic feature in all forms of the disease. The incubation period varies from 3 to 5 days. In peracute cases, sudden death without clinical signs may be observed (Carter & De Alwis, 1989; De Alwis, 1992). Water buffaloes are generally more susceptible to HS than cattle and show more severe forms of disease with profound clinical signs. Severe subcutaneous oedema of the mandible, neck and brisket is a distinctive feature of the disease. In endemic areas, mortality is largely confined to older calves and young adults.

At post-mortem, most animals succumbing to HS typically show marked swelling of the neck caused by severe blood-tinged oedema. There are also abundant petechial haemorrhages in many tissues and organs, particularly in serosal membranes. The thoracic, pericardial and abdominal cavities may contain sero-sanguinolent fluid. The lungs are notably congested and oedematous, and foam is generally present in the nasal cavity, trachea and bronchi. Microscopically, there is interstitial pneumonia and pulmonary oedema as well as focal infiltrates of neutrophils and macrophages in many tissues. All these lesions are similar to those observed in severe sepsis and septic shock.

Massive epizootics may occur in endemic as well as non-endemic areas (Carter & De Alwis, 1989; Chanda *et al.*, 2024; De Alwis, 1992). Within an endemic country, there can be spatial and temporal variation in the occurrence of HS outbreaks (Chanda *et al.*, 2024). This indicates the need for targeted and tailored prevention and control strategies within endemic settings. HS has been identified as a secondary complication in cattle and water buffaloes following outbreaks of foot and mouth disease (FMD). Case fatality approaches 100% if treatment is not followed at the initial stage of infection (Carter & De Alwis, 1989; De Alwis, 1992). Appropriate antibiotics need to be selected following antimicrobial susceptibility tests for effective reduction of case fatality as multidrug resistant *P. multocida* strains are emerging (Prajapati *et al.*, 2020a; Shivachandra *et al.*, 2011). The ability of *P. multocida* to form biofilms has been documented in different serogroups including serotype B:2 (Prajapati *et al.*, 2020b), which are known to significantly enhance the resistance of *P. multocida* to antibiotics *in vivo*.

The diagnosis of the disease is based on the clinical signs, gross lesions, morbidity and mortality patterns. Confirmation requires the isolation and characterisation of the pathogen using conventional and molecular techniques. There are no confirmed reports of human infections with *P. multocida* B:2 and E:2; however, other serotypes from companion animals like cat and dog do cause human infections and precautions should be taken to avoid exposure. The organism 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 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)						
Bacterial isolation	□□□	□□□	☑□□	☑☑☑	☑□□	□□□
Antigen detection	□□□	□□□	☑□□	☑☑☑	☑□□	□□□
Mouse pathogenicity test	□□□	□□□	☑□□	☑☑☑ ^(h)	☑□□	□□□

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)
CIEP	□□□	□□□	☑□□	☑☑☑	☑□□	☑□□
Rapid slide agglutination test	□□□	☑□□	☑□□	☑☑☑	□□□	□□□
PCR	□□□	☑□□	☑□□	☑☑☑	☑□□	□□□
Real-time PCR	□□□	☑□□	☑□□	☑☑☑	☑□□	□□□
Detection of immune response						
IHA	□□□	☑□□	☑□□	☑☑□	☑□□	~~☑☑☑~~ ☑☑□
<u>ELISA</u>	<u>□□□</u>	<u>☑□□</u>	<u>☑□□</u>	<u>□□□</u>	<u>☑☑□</u>	<u>☑☑□</u>

Key: ☑☑☑ = recommended for this purpose; ☑☑□ = recommended but has limitations;

☑□□ = suitable in very limited circumstances; □□□ = not appropriate for this purpose.

CIEP = counter-immuno-electrophoresis; PCR = polymerase chain reaction;

IHA = indirect haemagglutination; ELISA = enzyme-linked immunosorbent assay.

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

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

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

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

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

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

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

^(h)For animal welfare/ethical reasons, this test should only be used as a last resort.

1. Isolation and detection of the agent

1.1. Cultural and biochemical methods

True septicaemia in HS occurs at the terminal stage of the disease, therefore blood samples and nasal swabs/discharge should be taken from sick animals immediately before death. Animals in the early stages of the disease may not contain *P. multocida* in blood. The bacteria are also not consistently present in the nasal secretions or body fluids of sick animals. Blood samples collected from ailing animals undergoing antibiotic treatment may not indicate the presence of bacteria due to their suppression in the circulation.

A blood sample or swab collected from the heart is satisfactory only if taken immediately after death. If the animal has been dead for a long time, bone marrow from a long bones (humerus, femur, tibia, etc.) can be used for bacterial isolation. If there is no facility for post-mortem examination, blood can be collected from the jugular vein by incision or aspiration. Blood samples in any standard transport medium should be dispatched on ice and well packed to avoid any leakage. If the blood samples are not transported to the laboratory within a few hours, they can be frozen. However repeated freezing and thawing may destroy the organism and is not advisable.

Blood smears from affected animals are stained with Gram, Leishman's or methylene blue stains. The organisms appear as Gram-negative, bipolar-staining short bacilli. No conclusive diagnosis can be made on the basis of direct microscopic examinations alone.

Blood samples, or swabs eluted into 2–3 ml sterile physiological saline, are cultured. Alternatively, the surface of a long bone is disinfected with alcohol and split open. The marrow is extracted aseptically and cultured. Direct culture is usually satisfactory only if the material is fresh and free from contaminants or post-mortem invaders that would otherwise overgrow any *Pasteurella* present.

For biological examinations, a small volume (0.2 ml) of eluted nasal/blood swabs or a portion of tissues/bone marrow in saline is inoculated subcutaneously or intramuscularly into mice. The mouse usually serves as a biological 'screen' for extraneous organisms. If viable *P. multocida* is present, the mice die 24–36 hours following inoculation, and a pure growth of *P. multocida* can be seen in blood smears. Pure cultures of *P. multocida* can usually be grown from blood (preferably from heart) of the mice, even when the original samples (nasal swab and tissue samples) come from relatively old carcasses. The organism can be identified by its morphological and cultural characteristics, biochemical reactions and serological tests. The mouse inoculation method may be used as a last resort in isolation of bacterial agent, particularly from heavily contaminated nasal/tissue materials.

A suitable bacteriological medium for *Pasteurella* is casein/sucrose/yeast (CSY) agar containing 5% blood. The composition of this medium is casein hydrolysate (3 g), sucrose (3 g), yeast extract (5 g), sodium chloride (5 g), anhydrous dipotassium hydrogen orthophosphate (3 g), and distilled water to 1 litre. The pH is adjusted to 7.3–7.4, after which 1.5% agar is added. The medium is autoclaved at 1 bar for 15 minutes. After cooling to 45–50°C, 5% calf blood (antibody-free *P. multocida*) is added (Wijewardana *et al.*, 1986). Conventional blood agar may also be used.

Freshly isolated *P. multocida* forms smooth, greyish glistening translucent colonies, approximately 1 mm in diameter, on blood agar after 24 hours' incubation at 37°C. Colonies grown on CSY agar are larger. Old cultures, particularly those grown on media devoid of blood, may produce smaller colonies. *Pasteurella multocida* does not grow on MacConkey agar. Gram-stained blood or tissue smears show Gram-negative, short, ovoid, bipolar-staining coccobacilli. A degree of pleomorphism will be noted, particularly in old cultures, with longer rods of varying length. The bipolar staining will be more evident with methylene blue or Leishman's stain.

HS organisms produce oxidase, catalase and indole, and will reduce nitrates. They do not produce hydrogen sulphide or urease, and fail to use citrate or liquefy gelatin. Glucose and sucrose are always fermented with the production of acid only. Most strains also ferment sorbitol. Some strains ferment arabinose, xylose and maltose, whereas salicin and lactose are almost invariably not fermented.

One property of HS-causing strains of *P. multocida* is the ability to produce the enzyme hyaluronidase (Carter & Chengappa, 1980). Having identified the genus and species by cultural characteristics and biochemical tests, hyaluronidase production may then be used as a specific test for HS-causing pasteurellae. It should be noted that B serotypes other than B:2 (or 6:B), and type E, are hyaluronidase negative.

A hyaluronic-acid-producing culture is streaked across the centre of a dextrose starch agar plate. The *Pasteurella* culture to be tested for hyaluronidase production is streaked at right angles. The plates are incubated at 37°C for 18 hours. Originally, hyaluronic-acid-producing *Streptococcus equi* was used, but a convenient culture for this purpose is a capsulated mucoid *P. multocida* type A culture. At the point of intersection, the mucoid growth of the hyaluronic acid producer will diminish into a thin line of growth, indicating the production of hyaluronidase by the test culture. Use of freshly prepared plates and a humidified incubator will facilitate hyaluronic acid production and, thereby, the interpretation of the test.

1.2. Serotyping methods

Several serotyping tests are used for the identification of the HS-causing serotypes of *P. multocida*. These consist of a rapid slide agglutination test (~~Namioka & Murata, 1961a~~), an indirect haemagglutination (IHA) test for capsular typing (Carter, 1955), an agglutination test using hydrochloric-acid-treated cells for somatic typing (~~Namioka & Murata, 1961b~~), the agar gel immunodiffusion (AGID) test (~~Anon, 1981~~; Heddleston *et al.*, 1972; Wijewardana *et al.*, 1982), and the counter immunoelectrophoresis test (CIEP) (Carter & Chengappa, 1981).

Hyperimmune antisera for most of these tests are prepared against specific reference strains in rabbits. Cultures in CSY broth (6–8-hours old) are seeded on to CSY blood agar medium. After overnight incubation (18–20 hours) the growth is washed into physiological saline containing 0.3% formalin. The turbidity of the cell suspension is adjusted to that of MacFarland's tube No. 4. Rabbits are inoculated intravenously at 3–4-day intervals with 0.2, 0.5, 1.0, 1.5 and finally, 2.0 ml of this suspension. The rabbits are inoculated subcutaneously or intramuscularly 1 week after the last injection with 0.5 ml of a similar, but live, suspension. The animals are bled 10 days later. The serum is stored at –20°C, but small quantities for regular use are stored at 4°C with the addition of 1/10,000 merthiolate.

1.2.1. Rapid slide agglutination test (capsular typing)

A single colony is mixed with a drop of saline on a slide, a drop of antiserum is added, and the slide is warmed gently. A coarse, floccular agglutination appears within 30 seconds. Old cultures may give a fine, granular agglutination that takes longer to appear.

1.2.2. Indirect haemagglutination test (capsular typing)

This was originally performed using antigen-sensitised human type 'O' red blood cells (RBCs) (Carter, 1955), but more recently sheep RBCs have been used (Sawada *et al.*, 1985; Wijewardana *et al.*, 1986). The antigen is prepared as follows:

A 6–8-hour broth culture of a reference strain is seeded on to CSY blood agar plates and incubated overnight at 37°C. The growth is harvested in 3 ml physiological saline containing 0.3% formalin. This suspension is then heated at 56°C for 30 minutes, centrifuged at 3000 *g* for 15 minutes at 4°C, and the clear supernatant fluid is stored at –20°C. If a refrigerated centrifuge is not available, centrifugation at 1500 *g* for 30 minutes gives a supernatant fluid. This is used as the antigen extract. A similar procedure is followed for preparing an antigen extract from an unknown strain that is to be typed.

Sheep blood is collected aseptically into an anticoagulant and centrifuged at 500 *g* for 10 minutes. The packed RBCs are washed three times in sterile physiological saline. The antigen extract from an unknown strain prepared by the method described above is used to sensitise the RBCs or absorbed on to the RBCs. This is done by adding 15 volumes of the antigen extract to the RBCs and incubating the mixture for 1 hour at 37°C with frequent shaking. The sensitised RBCs are recovered by centrifugation, washed three times in sterile physiological saline, and made up to a final 1% suspension in physiological saline. The type-specific hyperimmune antiserum (three volumes) is absorbed by the addition of packed RBCs (one volume) for 30 minutes at room temperature, and then centrifuged at 500 *g* for 10 minutes to pellet the RBCs. The absorbed antiserum is then inactivated by heating at 56°C for 30 minutes.

The test itself can be carried out in tubes or plates, and is performed in two rows. The test described below is for standard microtitre plates.

- i) The capsular extract of the unknown strain is prepared as described above and used to sensitise the sheep RBCs. The known type-specific hyperimmune sera raised in rabbits against types A, B, D and E are diluted as follows:
- ii) Using four separate rows of wells, the first wells are filled with 0.72 ml saline followed by 0.4 ml in the next six wells or more.
- iii) The type-specific hyperimmune sera are each separately diluted in each row by adding 0.08 ml of the serum to the first well and mixing with a pipette. From this well 0.4 ml is transferred to the next well, mixed, and the process carried on until well seven. This constitutes 1/10 dilution in the first well and a doubling dilution thereafter.
- iv) All the wells are each filled with 0.4 ml of antigen-adsorbed/sensitised RBCs, shaken slightly and left at room temperature. By the addition of the sensitised blood, the serum dilutions in the wells are doubled, i.e. 1/20 in well one, 1/40 in the second, and so on. A positive, negative and saline control are included in each test run.
- v) The first reading is taken after 2 hours and a final reading after 18 hours. A coarse agglutination of the RBCs along the sides of the concave wells is taken as a positive reading, and the formation of a button at the centre of the wells as negative. An arbitrary score of 1 to 4 is given depending on the size of the agglutination. An unknown strain is identified with the hyperimmune serum that has agglutination. In the absence of agglutination with all sera, the strain is considered to be untypeable.

While IHA can be used for typing unknown strains, the test itself is more efficient when dealing with serotypes B and E and is more reliable as a quantitative test against these strains.

1.2.3. Agar gel immunodiffusion tests

AGID tests are used for what is described as 'capsular' as well as 'somatic' typing, depending on the antigens and antisera used. The double-diffusion technique is employed. Wells are punched in the solid agar in a circular pattern with one centre well surrounded by six peripheral wells.

- i) *Capsular typing*: The gel medium is 1.0% Noble agar, or equivalent product, in 0.2 M phosphate buffer containing merthiolate at a final concentration of 1/10,000 (Anon, 1984;

Wijewardena *et al.*, 1982). Antigens and antisera are the same as for capsular typing by the IHA method (Carter, 1955). The standard antiserum is placed in the centre well, and the test antigens are placed in the peripheral wells alternately with standard homologous antigen.

- ii) *Somatic typing*: The gel medium consists of special Noble agar, or equivalent product, at a concentration of 0.9% in 0.85% sodium chloride solution.
- iii) For antigen preparation, the growth from each plate is harvested in 1 ml of 8.5% sodium chloride containing 0.3% formalin. The suspension is heated at 100°C for 1 hour, the cells are sedimented by centrifugation, and the supernatant fluid is used as antigen.
- iv) Antisera against 16 somatic types (Heddleston *et al.*, 1972) are prepared in chickens. Oil-emulsified bacterin¹ (1 ml) is injected subcutaneously into the mid-portion of the neck of 12–16-week-old male birds. A further injection is made 3 weeks later of 1 ml intramuscularly into the breast, 0.5 ml on each side of the sternum. The birds are bled 1 week later, and the serum is separated and preserved with 0.01% thiomersal and 0.06% phenol. Sera are tested against all somatic types and sera that cross-react are discarded. Some antisera preparations against B:2 may cross react with the somatic type 5.
- v) The test antigen is placed in the centre well and antisera against the different serotypes are placed in the peripheral wells. All HS serotypes (Asian and African) will react with type 2 antiserum. Cross-reactions may occur with type 5.

1.2.4. Counter immunoelectrophoresis

CIEP offers a rapid method for the identification of capsular types B and E cultures.

- i) *Preparation of capsular substance*

Capsular substance is prepared in the same manner as described for the IHA test.

- ii) *Preparation of hyperimmune antisera*

Antisera are prepared in rabbits as for the IHA test.

- iii) *Medium for CIEP*

The medium for the CIEP consists of agarose (2.0 g), barbitone sodium (2.06 g), diethyl barbituric acid (0.37 g), distilled water (180 ml), and 1/1000 merthiolate (20 ml).

- iv) *Veronal acetate buffer (barbitone buffer)*

The barbitone buffer consists of barbitone sodium (29.24 g), anhydrous sodium acetate (11.70 g), 0.1 N hydrochloric acid (180 ml), and distilled water to 3 litres. The pH should be 8.8.

- v) *Preparation of slides*

The electrophoresis plates are prepared by precoating glass slides (57 mm × 70 mm) with 12 ml volumes of the medium. Seven wells, 4 mm in diameter and 7 mm apart, are cut in a row. A parallel set of wells is cut 6 mm (centre to centre) away from the other set of wells.

- vi) *Test procedure*

The well on the side of the cathode is loaded with a 20 µl volume of capsular antigen, while an equal volume of type-specific antiserum is loaded on to the well on the side of the anode. Controls included in the test are 0.85% sodium chloride solution against positive antiserum, and capsular extract against negative rabbit serum as well as positive and negative control samples. The electrophoresis tank is filled with barbitone buffer, pH 8.8. The antigen and antiserum are electrophoresed for 30 minutes at 150 V (25 V/cm). The plates are then examined for precipitation lines.

¹ The bacterial antigens in broth are covered by a light mineral oil (adjuvant) and then emulsified (stabilised) with an emulsifying agent, in this case lanolin or lanoline (wool fat). This has to be done as the watery phase with the bacteria (broth) will not mix with the oily phase (adjuvant). The proportion of oil to emulsifying agent will vary with different batches of lanolin and will have to be adjusted accordingly. The higher the percentage of lanoline, the higher the stability of the emulsion.

vii) Interpretation of the results

The presence of a distinct line between the antigen and antiserum wells is considered to be a positive result.

1.2.5. Agglutination tests (somatic antigen)

The somatic 'O' antigen is prepared by a method similar to that described previously for the IHA test (Namioka, 1978; ~~Namioka & Murata, 1961b~~). A 6–8-hour test culture is seeded on to CSY blood agar and incubated overnight. The growth is harvested in 2–3 ml of physiological saline containing 0.3% formalin per plate, and centrifuged at 3000 *g* for 15 minutes at 4°C (or 1200–1500 *g* for 30–45 minutes at room temperature). The deposited bacteria are resuspended in 25 ml normal HCl saline (0.85% saline in a normal HCl solution) to give an opacity approximately equivalent to Brown's opacity tube No. 6, and incubated overnight. The suspension is again centrifuged, the supernatant fluid is discarded, and the cell residue is washed three times successively in phosphate buffered saline (PBS) at pH 5.0, 6.0 and 7.0, respectively.

Finally, a suspension of the residual cells, equivalent to Brown's opacity tube No. 6, is prepared in PBS at pH 7.0. Any suspensions that autoagglutinate should be discarded.

Antisera are prepared against whole bacterial cell suspensions of the reference strains B:2 (Asian HS), E:2 (African HS) and 11:B (Australian 989, non-HS). Agglutination tests are carried out on a slide and the test antigen is used against the three types of sera. A fine granular agglutination indicates a specific somatic agglutination. Tests carried out against the standard antigens will facilitate reading and interpretation. When nonspecific partial agglutination occurs, the tests carried out with tenfold dilutions of the serum against the test and standard antigens will help to identify somatic antigen.

1.2.6. Serotype designation

Broadly, two typing systems are adopted. One is 'capsular' typing by Carter's IHA test (Carter, 1955) or by AGID tests (~~Anon, 1984~~; Wijewardena *et al.*, 1982). The other is 'somatic' typing by the method of Namioka & Murata (Namioka, 1978; ~~Namioka & Murata, 1961b; 1961c~~), and by the method of Heddleston *et al.* (1972). It is generally agreed that designation of serotypes should be based on a somatic–capsular combination. Two systems commonly in use are the Namioka–Carter and the Carter–Heddleston systems. In the former system, Asian and African HS serotypes are designated 6:B and 6:E, respectively, while in the latter system they are designated B.2 and E.2, respectively.

1.3. Molecular methods – detection of nucleic acids ~~Nucleic acid recognition methods~~

Extraction of nucleic acids

Different kits and procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid is important and can be checked using a suitable method as appropriate to the circumstances.

1.3.1. Real-time PCR

Primers and probes (sequence)

<u>Pathogen/ target gene</u>	<u>Primer/probe (5'–3')</u>	<u>Concentration</u>	<u>Cycling parameters^(a)</u>
<u>Method 1: HS-est-RT-PCR; Petersen <i>et al.</i>, 2014; GenBank Accession No.: 178 bp</u>			
<u>est</u>	<u>Fwd name: PmHSE: CCT-TCT-ACA-CAA-GST-GGT-TTG-A</u> <u>Rev name: Pm HSAR: GCC-AAR-TCA-TTA-CCR-TCT</u>	<u>0.3 mM</u> <u>0.3 mM</u>	<u>40 cycles of: 95°C/30 sec</u> <u>and 50°C/60 sec</u>

^(a)A denaturation step prior to cycling has not been included.

1.3.2. Conventional PCR

Primers (sequences)

Pathogen/ target gene	Primer (5'–3')	Concentration	Cycling parameters ^(a)
Method 1: <i>Pasteurella multocida</i>-specific PCR assay; Townsend <i>et al.</i>, 1998; GenBank Accession No.: AF016259; amplicon size: 460 bp			
<u>Kmt1</u>	Fwd name: KMT1T7: ATC-CGC-TAT-TTA-CCC-AGT-GG Rev name: KMT1SP6: GCT-GTA-AAC-GAA-CTC-GCC-AC	3.2 pmol 3.2 pmol	30 cycles of: 95°C/1 min and 55°C/1 min; final extension at 72°C/9 min
Method 2: HS-causing type-B-specific PCR assay; Townsend <i>et al.</i>, 1998; GenBank Accession No.: AF016260; amplicon size: 620 bp			
<u>Cap B</u>	Fwd name: KTT72: AGG-CTC-GTT-TGG-ATT-ATG-AAG Rev name: KTSP61: ATC-CGC-TAA-CAC-ACT-CTC	3.2 pmol 3.2 pmol	30 cycles of: 95°C/1 min and 55°C/1 min; final extension at 72°C/9 min
Method 3: <i>Pasteurella multocida</i> multiplex capsular PCR typing system; Townsend <i>et al.</i>, 2001; amplicon sizes: 1044 bp, 760 bp, 657 bp, 511 bp and 851 bp			
<u>Cap A</u>	Fwd name: CAPA-FWD: TGC-CAA-AAT-CGC-AGT-CAG Rev name: CAPA-REV: TTG-CCA-TCA-TTG-TCA-GTG	3.2 pmol 3.2 pmol	[Amplicon size: 1044 bp] 30 cycles of: 95°C/1 min and 55°C/1 min; final extension at 72°C/5 min
<u>Cap B</u>	Fwd name: CAPB-FWD: CAT-TTA-TCC-AAG-CTC-CAC-C Rev name: CAPB-REV: GCC-CGA-GAG-TTT-CAA-TCC	3.2 pmol 3.2 pmol	[Amplicon size: 760 bp] 30 cycles of: 95°C/1 min and 55°C/1 min; final extension at 72°C/5 min
<u>Cap D</u>	Fwd name: CAPD-FWD: TTA-CAA-AAG-AAA-GAC-TAG-GAG-CCC Rev name: CAPD-REV: CAT-CTA-CCC-ACT-CAA-CCA-TAT-CAG	3.2 pmol 3.2 pmol	[Amplicon size: 657 bp] 30 cycles of: 95°C/1 min and 55°C/1 min; final extension at 72°C/5 min
<u>Cap E</u>	Fwd name: CAPE-FWD: TCC-GCA-GAA-AAT-TAT-TGA-CTC Rev name: CAPE-REV: GCT-TGC-TGC-TTG-ATT-TTG-TC	3.2 pmol 3.2 pmol	[Amplicon size: 511 bp] 30 cycles of: 95°C/1 min and 55°C/1 min; final extension at 72°C/5 min
<u>Cap F</u>	Fwd name: CAPF-FWD: AAT-CGG-AGA-ACG-CAG-AAA-TCA-G Rev name: CAPF-REV: TTC-CGC-CGT-CAA-TTA-CTC-TG	3.2 pmol 3.2 pmol	[Amplicon size: 851 bp] 30 cycles of: 95°C/1 min and 55°C/1 min; final extension at 72°C/5 min
Method 4: <i>Pasteurella multocida</i> type A specific PCR; Gautam <i>et al.</i>, 2004; GenBank Accession No.: AF067175; amplicon size: 564 bp			
<u>Cap A</u>	Fwd name: RGPMA5: AAT-GT-TTG-CGA-TAG-TCC-GTT-AGA Rev name: RGPMA6: ATT-TGG-CGC-CAT-ATC-ACA-GTC	20 pmol 20 pmol	30 cycles of: 95°C/ 45 sec and 56°C/45 sec; final extension at 72°C/6 min
Method 5: <i>Pasteurella multocida</i> Multiplex LPS PCR Typing System; Harper <i>et al.</i>, 2015; GenBank Accession No.: HQ162664–HQ162679; amplicon sizes: varying: 1307 bp, 810 bp, 474 bp, 550 bp, 1175 bp, 1668 bp, 931 bp, 255 bp			
<u>L1 (Heddleston Sero var 1 and 14)</u>	Fwd name: BAP6119: ACA-TTC-CAG-ATA-ATA-CA-CCCG Rev name: BAP6120: ATT-GGA-GCA-CCT-AGT-AAC-CC	0.4 µM 0.4 µM	[Amplicon size: 1307 bp] 30 cycles of: 96°C/30 sec and 52°C/30 sec; final extension at 72°C/5 min
<u>L2 (Heddleston Sero var 2/5)</u>	Fwd name: BAP6121: CTT-AAA-GTA-ACA-CTC-GCT-ATTGC Rev name: BAP6122: TTT-GAT-TTC-CCT-TGG-GAT-AGC	0.4 µM 0.4 µM	[Amplicon size: 810 bp] 30 cycles of: 96°C/30 sec and 52°C/30 sec; final extension at 72°C/5 min
<u>L3 (Heddleston Sero var 3 and 4)</u>	Fwd name: BAP7213: TGC-AGG-CGA-GAG-TTG-ATA-AAC-CAT-C Rev name: BAP7214: CAA-AGA-TTG-GTT-CCA-AAT-CTG-AAT-GGA	0.4 µM 0.4 µM	[Amplicon size: 474 bp] 30 cycles of: 96°C/30 sec and 52°C/30 sec; final extension at 72°C/5 min

<u>L4 (Heddleston Serovar 6 and 7)</u>	Fwd name: BAP6126: CTT-TAT-TTG-GTC-TTT-ATA-TAT-ACC Rev name: BAP6125: TTT-CCA-TAG-ATT-AGC-AAT-GCC-G	0.4 µM 0.4 µM	[Amplicon size: 550 bp] 30 cycles of: 96°C/30 sec and 52°C/30 sec; final extension at 72°C/5 min
<u>L5 (Heddleston Serovar 9)</u>	Fwd name: BAP6129: AGA-TTG-CAT-GGC-GAA-ATG-GC Rev name: BAP6130: CAA-TCC-TCG-TAA-GAC-CCC-C	0.4 µM 0.4 µM	[Amplicon size: 1175 bp] 30 cycles of: 96°C/30 sec and 52°C/30 sec; final extension at 72°C/5 min
<u>L6 (Heddleston Serovar 10, 11, 15, and 12)</u>	Fwd name: BAP7292: TCT-TTA-TAA-TTA-TAC-TCT-CCC-AAG-G Rev name: BAP7293: AAT-GA-AGG-TTT-AAA-AGA-GAT-AGC-TGG-AG	0.4 µM 0.4 µM	[Amplicon size: 1668 bp] 30 cycles of: 96°C/30 sec and 52°C/30 sec; final extension at 72°C/5 min
<u>L7 (Heddleston Serovar 8 and 3)</u>	Fwd name: BAP6127: CCT-ATA-TTT-ATA-TCT-CCT-CCC-C Rev name: BAP6128: CTA-ATA-TAT-AAA-CCA-TCC-AAC-GC	0.4 µM 0.4 µM	[Amplicon size: 931 bp] 30 cycles of: 96°C/30 sec and 52°C/30 sec; final extension at 72°C/5 min
<u>L8 (Heddleston Serovar 16)</u>	Fwd name: BAP6133: GAG-AGT-TAC-AAA-AAT-GAT-CGG-C Rev name: BAP6134: TCC-TGG-TTC-ATA-TAT-AGG-TAG-G	0.4 µM 0.4 µM	[Amplicon size: 255 bp] 30 cycles of: 96°C/30 sec and 52°C/30 sec; final extension at 72°C/5 min

^(a)A denaturation step prior to cycling has not been included.

1.3.3. Loop-mediated isothermal amplification (LAMP) assays

<u>Pathogen/ target gene</u>	<u>Primer/probe (5'–3')</u>	<u>Concentration</u>	<u>Cycling parameters^(a)</u>
Method 1: Pm-LAMP assays; Moustafa & Bennett, 2017; GenBank Accession No.: AF016259			
<u>Kmt1</u>	Pm-AMM-F3: CAG-CAA-TTT-CGA-GCA-AAC-AAT-G Pm-AMM-B3: AGC-CAA-ATA-AAA-GAC-TAC-CGA-C Pm-AMM-FIP: CAC-CGC-CCC-ACT-GGG-TAA-ATA-GTG-GGG-CTT-TAC-GCT-GAT Pm-AMM-BIP: ATG-AAC-CGA-TTG-CCG-CGA-AAT-TGA-AGC-CCA-CTC-ACA-ACG-AG Pm-AMM-LF: CGG-ATA-GAG-CAG-TAA-TGT-CAG-CA Pm-AMM-LB: TGC-CAC-TTG-AAA-TGG-GAA-ATG	0.314 µmol/l for each of FIP and BIP. 0.157 µmol/l for each of LB and LF. 0.029 µmol/l for each of F3 and B3	63°C for 34 minutes
Method 2: HS LAMP assay. Moustafa & Bennett, 2017; GenBank Accession No.: JQAO00000000			
<u>CapB</u>	1517-AMM-F3: AGC-AAT-GAA-TGT-TGC-TGC 1517-AMM-B3: TTG-TTG-TAT-CGG-GTT-GTT-TG 1517-AMM-FIP: ACT-GTT-GAA-CCG-AGA-TAA-AAC-TCA-ATT-GCC-AAA-AGA-AAT-GGG- GT 1517-AMM-BIP: CCA-TTG-TAA-AGT-GGA-TGC-TAC-TCG-GTT-TCC-TGC-ACC-ACC-TAA 1517-AMM-LB: CGT-AGG-TCA-TAA-TGG-CAT-TGG-TGTG	0.29 µmol/l for each of FIP, BIP. 0.143 µmol/l for LB. 0.029 µmol/l for each of F3 and B3	63°C for 28 minutes

General notes:

- PCR technology can be applied for rapid, sensitive and specific and/or detection of ≤10 *P. multocida* organisms per reaction directly using a colony, culture, infected tissue or mixed culture following lysate preparation.
- Pasteurella multocida* can be detected regardless of the purity of the specimen, an advantage if the specimen is from an old carcass or from tonsil or nasal swabs. The swab should be inoculated in 2 ml CSY broth and incubated on a roller for 2–4 hours; 2 µl of the culture is then added directly to the PCR mixture prior to amplification.
- For each colony PCR, material from two to three well-isolated *P. multocida* colonies is collected using a sterile tip on a 20 µl micropipette (volume set at 20 µl) inserted into the middle of each colony. The collected material is then added to a 50 µl PCR mixture and mixed thoroughly by pipetting.
- Species-specific PCR can identify all subspecies of *P. multocida*, but has been found to give a false positive with both *P. avium* biovar 2 and *P. canis* biovar 2 (Method 1).

- v) For capsular serotyping, multiplex capsular PCR typing system could be employed along with species-specific PCR assay (Method 3).
- vi) For somatic serogrouping, multiplex LPS PCR typing system could be employed along with a species-specific PCR assay (Method 5).
- vii) Presumptive capsular and somatic serotypes could be obtained within 3–4 hours, in comparison with biochemical analysis and conventional serotyping, which can take up to 2 weeks.
- viii) For setting up the PCR, use a reaction mix of either 25 µl of 50 µl containing 1 × PCR buffer, 200 µM each deoxynucleotide triphosphate (dNTP), 2 mM MgCl₂, 1 u *Taq* DNA polymerase or master mix, using either purified DNA, culture lysate or direct colony as template DNA.
- ix) For all PCR conditions, an initial denaturation step at 95°C for 5 minutes and final extension step at 72°C for 7 minutes needs to be included (Methods 1–5).
- x) Generally, it is suggested to optimise the reaction mix/reagents/template DNA in a thermocycler used to get desired amplicons, as it was found to vary in amplicon intensity between uniplex or multiplex PCR assays with combination of multiple pair of primers for capsular and somatic typing.
- xi) Upon amplifications, 5 µl of each sample is electrophoresed on a 2% agarose gel in 1 × Tris-acetate running buffer (TAE) at 4 V/cm for 1 hour. The gel is stained with 1% ethidium bromide and DNA fragments are viewed by UV transillumination (Methods 1–5).
- xii) For rapid and real-time diagnosis, the HS-est real-time PCR could be employed for detection of the specific types of *P. multocida* involved in HS compared with other PCR assays.
- xiii) Pm-LAMP assay and an HS-LAMP assay specific for serogroup B:2 strains associated with HS in cattle and buffalo could be used at field level as a simple alternate method to PCR assays, which are sophisticated laboratory-based methodologies.

1.3.1. *Pasteurella multocida*-specific PCR assay

PCR technology can be applied for rapid, sensitive and specific and/or detection of *P. multocida* (Miflin & Blackall, 2001; Townsend *et al.*, 1998a). The rapidity and high specificity of two of the *P. multocida*-specific assays (Miflin & Blackall, 2001; Townsend *et al.*, 1998a) provide optimal efficiency without the need for additional hybridisation. The *P. multocida*-specific PCRs (Miflin & Blackall, 2001; Townsend *et al.*, 1998a) identify all subspecies of *P. multocida*. The Miflin & Blackall PCR (Miflin & Blackall, 2001) was described as giving a false positive with both *P. avium* biovar 2 and *P. canis* biovar 2, while the Townsend *et al.* PCR (1998a) gave a false positive with *P. canis* biovar 2 (it has not been tested against *P. avium* biovar 2). Recently, both *P. avium* biovar 2 and *P. canis* biovar 2 have been re-named as *P. multocida* (Christensen *et al.*, 2004) – meaning that both the Townsend *et al.* (1998a) and the Miflin & Blackall (Miflin & Blackall, 2001) PCR assays are now regarded as being specific for *P. multocida*. Some difficulties remain as it is now known that sucrose-negative *P. multocida*-like organisms from large cat bite wounds form two groups. While both are positive in the Miflin & Blackall *P. multocida*-specific PCR (2001) only one group has been confirmed as true *P. multocida* by other genotypic methods (Christensen *et al.*, 2005). The Townsend *et al.* (1998a) PCR is described in the following paragraph.

A fraction of an isolated colony of the suspect organism is transferred directly into the PCR mixture. Alternatively, template DNA can be obtained from 2 µl of either a mixed or pure broth culture. All currently used methods for the preparation of template DNA produce reproducible results with the KMT1 primers (Townsend *et al.*, 1998a), and allow detection of ≤10 organisms per reaction. The sensitivity and specificity of the *P. multocida*-specific PCR offer the most compelling argument for the use of PCR technology in laboratory investigation of suspected HS cases. *Pasteurella multocida* can be detected regardless of the purity of the specimen, an advantage if the specimen is from an old carcass or from tonsil or nasal swabs. In such cases, the swab should be inoculated in 2 ml CSY broth and incubated on a roller for 2–4 hours; 2 µl of the culture is then added directly to the PCR mixture prior to amplification.

Primer sequences (Townsend *et al.*, 1998a):

<i>P. multocida</i> -specific PCR:	KMT1T7	5'-ATC-CGC-TAT-TTA-CCC-AGT-GG-3'
	KMT1SP6	5'-GCT-GTA-AAC-GAA-CTC-GCC-AC-3'

PCR conditions:

Template DNA is added to the PCR mixture (total volume of 25 µl) containing 1 × PCR buffer, 200 µM each deoxynucleotide triphosphate (dNTP), 2 mM MgCl₂, 3.2 pmol of each primer and 0.5 u Taq DNA polymerase. Thermocycler parameters are as follows: initial denaturation at 95°C for 5 minutes; 30 cycles of 95°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute; with a final extension at 72°C for 7 minutes. 5 µl of each sample is electrophoresed on a 2% agarose gel in 1 × Tris-acetate running buffer (TAE) at 4 V/cm for 1 hour. The gel is stained with 1% ethidium bromide and DNA fragments are viewed by UV transillumination.

The PCR amplification yields a product of 460 bp.

1.3.2. *Pasteurella multocida* multiplex capsular PCR typing system

Identification of the genes involved in the biosynthesis of the *P. multocida* A:1 (Chung *et al.*, 1998) and B:2 (Boyce *et al.*, 2000) polysaccharide capsules provided the required information to determine the biosynthetic region of the remaining three serogroups (D, E, and F) (Boyce *et al.*, 2000). Moreover, with the use of serogroup specific multiplex PCR, conflicting results as regards to typing of some strains could be confirmed (Townsend *et al.*, 2001). With this knowledge, serogroup-specific sequences were identified for use as primers in a multiplex capsular PCR-typing system (Townsend *et al.*, 2001). The *P. multocida*-specific primers are included as an internal control for species identification. In the multiplex capsular PCR typing system, the amplicon band giving the typing result may be unclear. In such cases, removal of the *P. multocida*-specific primers (KMT1T7, KMT1SP6) from the mixture can improve the result.

Primer sequences (Townsend *et al.*, 2001):

Multiplex capsular PCR:	CAPA-FWD	5'-TGC-CAA-AAT-CGC-AGT-CAG-3'
	CAPA-REV	5'-TTG-CCA-TCA-TTG-TCA-GTG-3'
	CAPB-FWD	5'-CAT-TTA-TCC-AAG-CTC-CAG-C-3'
	CAPB-REV	5'-GCC-CCA-GAG-TTT-CAA-TCC-3'
	CAPD-FWD	5'-TTA-CAA-AAG-AAA-GAC-TAG-GAG-CCC-3'
	CAPD-REV	5'-CAT-CTA-CCC-ACT-CAA-CCA-TAT-CAG-3'
	CAPE-FWD	5'-TCC-GCA-GAA-AAT-TAT-TGA-CTC-3'
	CAPE-REV	5'-GCT-TGC-TGC-TTG-ATT-TTG-TG-3'
	CAPF-FWD	5'-AAT-CGG-AGA-ACG-CAG-AAA-TGA-G-3'
	CAPF-REV	5'-TTC-CGC-CGT-CAA-TTA-CTC-TG-3'
	KMT1T7	5'-ATC-CGC-TAT-TTA-CCC-AGT-GG-3'
	KMT1SP6	5'-GCT-GTA-AAC-GAA-CTC-GCC-AC-3'

Size of resulting fragments:

Serogroup A	CAPA-FWD/CAPA-REV	1044 bp
Serogroup B	CAPB-FWD/CAPB-REV	760 bp
Serogroup D	CAPD-FWD/CAPD-REV	657 bp
Serogroup E	CAPE-FWD/CAPE-REV	511 bp
Serogroup F	CAPF-FWD/CAPF-REV	851 bp

PCR conditions:

Template DNA is added to the PCR mixture (total volume of 25 µl) containing 1 × PCR buffer, 200 µM each deoxynucleotide triphosphate (dNTP), 2 mM MgCl₂, 3.2 pmol of each primer and 1 u Taq DNA polymerase. In the original publication (Townsend *et al.*, 2001) it is suggested to use a standard cycling programme as per *P. multocida*-specific PCR assay. However, the cycling programme should be optimised to and validated for the model of thermocycler in use. Agarose gel electrophoresis is as described above.

1.3.3. HS-causing type B-specific PCR assay

Presumptive identification of HS-causing type B-specific *P. multocida* is also possible by PCR amplification (Townsend *et al.*, 1998a). Type B cultures with the predominant somatic antigen being either type 2 or 5 are identified by the amplification of a ~620 bp fragment with the KTSP61 and KTT72 primers.

Primer sequences (Townsend *et al.*, 1998a):

HS-causing type B-specific PCR	KTT72	5'-AGG-CTC-GTT-TGG-ATT-ATG-AAG-3'
	KTSP61	5'-ATC-CGC-TAA-CAC-ACT-CTC-3'

Conditions for HS-causing type B specific PCR are as described for *P. multocida* specific PCR. The usefulness of these primers has been reported for identification of serogroup B strains.

HS-causing type B specific PCR primers can also be used in a multiplex PCR with the *P. multocida* specific primers, dramatically decreasing the time required for *P. multocida* detection and presumptive identification of the HS-serotype. Multiplex PCR conditions are as described above except that 3.2 pmol of each of the four primers and 1 u *Taq* DNA polymerase are used. The use of the multiplex *P. multocida* specific/HS-causing type B specific PCR on suspect organisms can confirm the identity and provide a presumptive serotype within 3–4 hours, in comparison with biochemical analysis and conventional serotyping, which can take up to 2 weeks.

1.3.4. *Pasteurella multocida* type A specific PCR

Primers useful for typing of serogroup A strains with several somatic types have been reported to be useful in specific identification of isolates (Gautam *et al.*, 2004):

Primers:

RGPM5: 5' AAT GT TTG CGA TAG TCC GTT AGA 3'

RGPM6: 5' ATT TGG CGC CAT ATC ACA GTC 3'

PCR conditions:

Template DNA (50 ng) is added to the PCR mixture (total volume of 25 µl) containing 1 × PCR buffer, 200 µM each dNTPs, 1.5 mM MgCl₂, 20 pmol of each primer and 1 unit *Taq* DNA polymerase. Standard amplification conditions are as follows: initial denaturation at 95°C for 5 minutes; 30 cycles of 95°C for 45 seconds, 56°C for 45 seconds, 72°C for 45 seconds, followed by final extension of 72°C for 6 minutes. Amplified products are separated by agarose gel electrophoresis (1.5% agarose gel) in 0.5 × TBE buffer at 5 v/cm for 2 hours.

The PCR amplification yields a product of 564 bp.

The test can be applied on direct culture, boiled cell lysate and infected tissues.

1.3.5. *Pasteurella multocida* multiplex LPS PCR typing system

Pasteurella multocida strains can be classified into 16 somatic or lipopolysaccharide (LPS) serovars (serotypes) using the Heddlestone gel diffusion precipitin test. The genes required for LPS assembly have been identified. The multiplex PCR method was developed for LPS molecular typing based on LPS outer core structure genes (Harper *et al.*, 2015):

Primers:

BAP6119: 5' ACA TTC CAG ATA ATA CA CCCC 3'

BAP6120: 5' ATT GGA GCA CCT AGT AAC CC 3'

BAP6121: 5' CTT AAA GTA ACA CTC GCT ATT GC 3'

BAP6122: 5' TTT GAT TTC CCT TGG GAT AGC 3'

BAP7213: 5' TGC AGG CGA GAG TTG ATA AAC CAT C 3'

BAP7214: 5' CAA AGA TTG GTT CCA AAT CTG AAT GGA 3'

BAP6126: 5' CTT TAT TTG GTC TTT ATA TAT ACC 3'

BAP6125: 5' TTT CCA TAG ATT AGC AAT GCC G 3'

BAP6129: 5' AGA TTG CAT GGC GAA ATG GC 3'

BAP6130: 5' CAA TCC TCG TAA GAC CCC C 3'

BAP7292: 5' TCT TTA TAA TTA TAG TCT CCC AAG G 3'

BAP7293: 5' AAT GA AGG TTT AAA AGA GAT AGC TGG AG 3'

BAP6127: 5' CCT ATA TTT ATA TCT CCT CCC C 3'

BAP6128: 5' CTA ATA TAT AAA CCA TCC AAC GC 3'

BAP6133: 5' GAG AGT TAC AAA AAT GAT CGG C 3'

BAP6134: 5' TGC TGG TTC ATA TAT AGG TAG G 3'

Size of resulting fragments:

L1 (Heddlestone Serovar 1 and 14)	BAP6119/ BAP6120	1307 bp
L2 (Heddlestone Serovar 2/5)	BAP6121/ BAP6122	810 bp
L3 (Heddlestone Serovar 3 and 4)	BAP7213/ BAP7214	474 bp
L4 (Heddlestone Serovar 6 and 7)	BAP6126/ BAP6125	550 bp
L5 (Heddlestone Serovar 9)	BAP6129/ BAP6130	1175 bp
L6 (Heddlestone Serovar 10, 11, 15, and 12)	BAP7292/ BAP7293	1668 bp
L7 (Heddlestone Serovar 8 and 3)	BAP6127/ BAP6128	931 bp
L8 (Heddlestone Serovar 16)	BAP6133/ BAP6134	255 bp

PCR conditions:

Each of the final multiplex LPS PCRs (50 µl final volume) is performed in 1×Taq polymerase buffer (10 mM Tris-HCl, 1.5 mM MgCl₂, 50 mM KCl) containing 0.4 µM each primer, 0.2 mM deoxynucleoside triphosphates (dNTPs), and 1.7 U Taq polymerase. For each colony PCR, material from 2 to 3 well-isolated *P. multocida* colonies is collected using a sterile tip on a 20 µl micropipette (volume set at 20 µl) inserted into the middle of each colony. The collected material is then added to a 50 µl PCR mixture and mixed thoroughly by pipetting. For PCR using genomic DNA, approximately 50 ng of column-purified DNA is added to each PCR mixture. All reaction mixtures are mixed briefly then centrifuged for 10 seconds at 13,000 g. For colony PCR, the cycling conditions are 96°C for 10 minutes, followed by 30 cycles of 96°C for 30 seconds, 52°C for 30 seconds, and 72°C for 2.5 minutes, with a final extension at 72°C for 5 minutes. For PCR using genomic DNA as the template, the cycling conditions are identical to those in the colony PCR except that the initial denaturation step at 96°C is reduced to 5 minutes. Amplified products are separated by 2% agarose gel electrophoresis.

1.3.6 Real-time PCR and loop-mediated isothermal amplification (LAMP) assays

HS-*est* real-time PCR based on *est* target gene has been developed (Petersen *et al.*, 2014). It specifically detects ST122, ST63, ST147 and ST162 associated with HS. However, it did not detect strains of ST151 with capsular type D isolated from pigs that were found positive with HS-causing type B-specific PCR assay. The HS-*est* real-time PCR represents a fast and specific detection of the specific types of *P. multocida* involved in HS compared with other PCR assays.

Recently, the development of a Pm-LAMP assay and an HS-LAMP assay specific for serogroup B:2 strains associated with HS in cattle and buffalo were reported (Moustafa & Bennett, 2017). The preliminary findings suggest that the HS-LAMP assay has high sensitivity and specificity for detection of HS-associated *P. multocida*. It is presumed that the Pm-LAMP method should be a useful diagnostic tool for rapid and visible detection of *P. multocida* from diverse clinical materials of various host species. However, it needs further evaluation directly on clinical specimens, along with its utility in the field as a simple alternate to PCR assays, which are sophisticated laboratory-based methodologies.

1.3.74. Genotypic differentiation of isolates

Once presumptive (or definitive) identification has been made, further differentiation of isolates can be achieved by genotypic fingerprinting methods. Molecular techniques such as ribotyping based on sequence analysis of the 16S rRNA gene, enterobacterial repetitive intragenic consensus (ERIC)-PCR, repetitive extragenic palindromic sequence PCR (REP-PCR), and/or multilocus sequence typing (MLST) analyses can more easily and accurately differentiate subspecies of *P. multocida*. Restriction endonuclease analysis (REA) with the enzymes *HhaI* and *HpaII* has proved useful for characterisation of type B HS-serotypes. Among 71 *P. multocida* capsule serogroup B isolates, 20 DNA fingerprint profiles were observed. With HS-causing serogroup strains, 13 unique profile among 54 isolates resembling the profile of the somatic serotype 2 reference strain have been reported (Wilson *et al.*, 1992). In contrast, while a single *HhaI* profile was observed among 13 serogroup E isolates, differentiation of these strains was possible following *HpaII* digestion. *HpaII* appear to generate finer subdivisions than those achieved with the use of *HhaI* (Wilson *et al.*, 1995). Ribotyping and large DNA separation by pulsed-field gel electrophoresis also provide useful discrimination of serogroup B and E *P. multocida* isolates (Townsend *et al.*, 1997a). Genetic diversity of HS-causing *Pasteurella multocida* strains of animal or bird origin could be obtained by sequence analysis of the 16S rRNA gene. A study in the United Kingdom using 79 field isolates recovered from various species revealed nineteen 16S rRNA types that clustered into two distinct phylogenetic lineages (Davies, 2004). On the other hand, sequence analyses of Indian isolates of *P. multocida* serogroup B from

different animal species did not reveal considerable variation (Dey *et al.*, 2007). Multilocus sequence typing (MLST), a sequence-based typing system based on seven housekeeping genes has been used to identify strain diversity of bovine isolates of *P. multocida* as well as isolates from other host species (Davies *et al.*, 2004; Hotchkiss *et al.*, 2011; Sarangi *et al.*, 2016; Subaaharan *et al.*, 2010). MLST of 55 isolates of *P. multocida* associated with HS found that the majority include sequence type (ST) 122 ($n = 50$), and rarely ST63, ST147 and ST162 in comparison with other members of the species isolated from different lesion types and hosts (Peterson *et al.*, 2014).

PCR fingerprinting is feasible for any laboratory with PCR capability, with several methods previously used for *P. multocida* differentiation. Random amplified polymorphic DNA (RAPD) analysis and arbitrarily primed PCR (AP-PCR), respectively, have been shown to be useful for epidemiological studies of *P. multocida* isolated from rabbits (Chaslus-Dancla *et al.*, 1996). Repetitive sequence PCR analysis of *P. multocida* has proved useful for discrimination of avian and swine HS causing isolates, although all HS-causing strains analysed demonstrated similar profiles (Townsend *et al.*, 1997b; 1998b). However, molecular variability among HS-causing strains of *P. multocida* belonging to serogroup B has been found recently. Using repetitive extragenic palindromic (REP)-PCR, enterobacterial repetitive intragenic consensus (ERIC)-PCR and single primer PCR, genotypic differentiation among five *P. multocida* serogroup B isolates have been reported (Biswas *et al.*, 2004; Prajapati *et al.*, 2020b). RAPD and AP-PCR analysis of HS-causing *P. multocida* isolates have not also been previously described reported with less discriminatory power.

In addition, virulence-associated genes (VAGs) profiling of *P. multocida* isolates have also been found to be useful for the differentiation of multiple strains prevalent among diverse host species (Prajapati *et al.*, 2020b). Virulence genotyping in epidemiological studies commonly targets genes encoding fimbriae and other adhesins (ptfA, fimA, hsf-1, hsf-2, pfhA, and tadD), toxins (toxA), iron acquisition proteins (exbB, exbD, tonB, hgbA, hgbB, fur, and tpbA), sialidases (nanB and nanH), hyaluronidase (pmHAS), outer membrane proteins (OMPs) (ompA, ompH, oma87, and plpB), and superoxide dismutases (sodA and sodC) (Prajapati *et al.*, 2020b; Sarangi *et al.*, 2015). *Pasteurella multocida* strains exhibit distinct virulence gene patterns depending on their virulence potential, capsular type, and host species. Additionally, VAG profiling has proven useful for differentiating *P. multocida* strains circulating among diverse host species (Prajapati *et al.*, 2020b).

Two distinct MLST schemes have been developed for the characterisation of *P. multocida* strains: the multihost MLST scheme and the Rural Industries Research and Development Corporation (RIRDC)-based MLST scheme. The multihost MLST scheme includes isolates from a diverse range of hosts, including birds, pigs, sheep, and cattle, and is based on seven housekeeping genes (*adk*, *aroA*, *deoD*, *gdhA*, *g6pd*, *mdh*, and *pgi*). In contrast, the RIRDC MLST scheme, originally designed for avian isolates is based on a different set of seven housekeeping genes (*adk*, *est*, *pmi*, *zwf*, *mdh*, *gdh*, and *pgi*) and is also widely used for typing *P. multocida* isolates from non-avian species. Both MLST schemes are publicly accessible at the *P. multocida* MLST database (<http://pubmlst.org/pmultocida/>). Notably, several studies have reported that the multihost MLST genotype ST44 (corresponding to RIRDC ST122) is predominantly associated with HS in South-East Asia (Prajapati *et al.*, 2022). Recently, phylogeny based on whole genome multilocus sequence typing (wgMLST), an extended concept of the traditional MLST, indicated the clustering of HS-causing strains against non-HS-causing strains (Prajapati *et al.*, 2022).

1.3.5. Whole genome sequence-based typing

With advancements in next generation sequencing (NGS) technologies, bacterial whole genome sequencing (WGS) has become more time and cost efficient. Whole genome assemblies of *P. multocida* strains from various countries and host species are publicly available on multiple data platforms, facilitating studies on species evolution, genomic dynamics, niche specialisation, and pathogenomics. The draft genomes of *P. multocida* serotype B:2 have been sequenced and analysed in endemic countries with the data publicly available in genome databases. The draft genome size has been reported to range from 2.3 to 2.4 Mb, with a GC content of approximately 40.5% and 2181–2307 coding sequences (CDS) (Prajapati *et al.*, 2022; Sarker *et al.*, 2018). WGS data of *P. multocida* have been used to determine capsular types, LPS genotypes, and MLST sequence types (Peng *et al.*, 2021; Prajapati *et al.*, 2023). Additionally, sequencing enables rapid identification of known and putative virulence genes within a genome. Several command-line and web-based tools have been developed for strain typing using assembled WGS data. For instance, genome-based MLST typing can be easily performed using the Genome Analysis Services of Center for Genomic Epidemiology (<https://www.genomicepidemiology.org/>). Recently, wgMLST revealed that HS-causing strains form a distinct cluster from non-HS strains, all belonging to sequence type 122 (Prajapati *et al.*, 2022). Comparative genomics and molecular phylogenetic

analyses are increasingly used to explore similarities and divergences among bacterial strains, providing insights into their clustering, transmission, and disease associations in relation to temporal, spatial, and clinical data. In a recent study (Prajapati *et al.*, 2022), the pan-genome analysis of 46 bovine-origin *P. multocida* strains identified 7872 genes, comprising 1133 core genes (14%), 2594 dispensable genes (33%), and 4145 unique genes (53%), suggesting niche-specific adaptation.

1.4. Biomarker-based method

Recently, a matrix assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) biomarker-based method was developed for differentiating HS-causing strains (B:2,5, E:2,5, and B:3,4) from non-HS strains of *P. multocida*. The developed assisted model, which used two peaks (6419 and 7729 m/z), accurately differentiated HS-causing strains of *P. multocida* from non-HS-causing strains, with 98.2% accuracy for non-HS strains, 100% accuracy for classic B:2,5 and E:2,5 HS-causing strains, and 84.4% accuracy for combined HS-causing strains (B:2,5, E:2,5, and B:3,4) with an overall accuracy of 96.9% (Maddock *et al.*, 2024).

2. Serological tests

Serological tests for detecting antibodies are not normally used for diagnosis. The IHA test can be used for this purpose, following a method broadly similar to that described for capsular typing above. High titres detected by the IHA test are indicative of recent exposure to HS. As HS is a disease that occurs mainly in animals reared under unsophisticated husbandry conditions, where disease-reporting systems are also poor, there is often considerable delay in notification of outbreaks. Deaths occur very suddenly and no carcasses are available for examination when notification is made. In such situations, high IHA titres from 1/160 up to 1/1280 or higher among in-contact animals surviving in affected herds, are indicative of recent exposure to HS for the purpose of diagnosis.

The enzyme-linked immunosorbent assay (ELISA) is a rapid, highly sensitive and specific serological test that has been employed to detect antibodies against HS in a limited study. Most of these studies employed different antigens of *P. multocida* such as sonicated or heat killed whole cell, and OMPs/IROMPs, which have been prepared for ELISA to detect specific antibodies in animals. However, variability in batch-to-batch antigen preparation for the assay and non-availability of single or multiple HS-specific immuno-dominant antigens have precluded the wider application of ELISA in routine sero-monitoring and or sero-surveillance.

C. REQUIREMENTS FOR VACCINES

1. Background

1.1. Rationale and intended use of the product

The three types of vaccines used against HS are bacterins, alum-precipitated vaccine (APV) and oil-adjuvanted vaccine (OAV). To provide sufficient immunity with bacterins, repeated vaccination is required. Administration of dense bacterins can give rise to shock reactions, which are less frequent with the APV and almost nonexistent with the OAV.

A live HS vaccine prepared using an avirulent *P. multocida* strain B:3,4 (Fallow deer strain) has been used for control of the disease in cattle and water buffaloes over 6 months of age in Myanmar since 1989. It is administered by intranasal aerosol application (Carter *et al.*, 1991; Myint *et al.*, 2005). The vaccine has been recommended by the Food and Agriculture Organization of the United Nations (FAO) as a safe and potent vaccine for use in Asian countries. However, there is no report of its use in other countries and killed vaccines are the only preparations in use by the countries affected with HS.

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 conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

A local isolate of *P. multocida* representing the prevalent serotype is used. A well-capsulated, stable culture that produces large colonies of approximately 2 mm in diameter on CSY blood agar must be maintained. Seed cultures should be stored as semisolid nutrient agar stab cultures at room temperature, or as lyophilised cultures.

A calf is infected with the culture, and, within 2–3 hours of its death, blood is collected aseptically from the heart and stored at –20°C in 1 ml aliquots. A fresh aliquot is used for each new batch of vaccine. It is permissible to subculture this aliquot once or twice, provided the colony size does not diminish. A blood aliquot is thawed, plated on to CSY blood agar, and the growth is tested for agglutinability by the appropriate antiserum on a slide. A good culture will give a coarse floccular agglutination in under 30 seconds. A poor culture will yield only a fine granular agglutination.

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

Seed lots must be shown to be:

- i) *Pure*: Free from adventitious agents.
- ii) *Safe*: Produce no adverse reaction in the target species when given as recommended.
- iii) *Efficacious*: Stimulate effective immunity as indicated by potency tests.

The necessary tests are described in Section C.2.2.4 below.

2.2. Method of manufacture

2.2.1. Procedure

For vaccine production, dense suspensions of bacteria are necessary. They should have a minimum bacterial content of 1.5 g dry weight per litre of suspension. There are two methods of producing dense suspensions. The first is to culture on solid medium in Roux flasks and harvest in formalinised physiological saline, by which means suspensions of any density can be achieved. This is laborious as each flask must be harvested separately and tested for purity. The second and recommended method is the use of a large vessel with aerated cultures in a medium that specifically supports *P. multocida*.

There are two types of aeration process – by vortexing and sparging. Sterile air is provided by a compressor. In vortex aeration, the culture is stirred by an impeller shaft operating in the air stream, whereas in sparging aeration, the air is dispersed through a sparger. Intermittent aeration seems to produce denser growth (Thomas, 1968). The more finely dispersed the air, the better is the bacterial growth. Vessels of 20–40 litres are usually employed, and incubation is at 37°C. In continuous culture systems, once a maximum density has been reached, usually within 15 hours, about 25% of the working volume is harvested and replaced hourly. The harvests of continuous cultures are collected in relatively small volumes into separate vessels, but, after several days, the density diminishes, presumably through loss of capsular antigen. For this reason, batch cultures are preferred. If batch culture vessels are inoculated at a rate of 50 ml/litre of medium, maximum turbidity is obtained within 15–18 hours, when the growth can be terminated by the addition of formalin to a final concentration of 0.5%. This procedure, where a large inoculum is employed and the growth is terminated within a short period, helps to minimise the chances of contamination. The turbidity is standardised against a reference containing the equivalent dry weight/volume of 1.5 g/litre.

Dense cultures are also obtained using fermenters, where heat sterilisation of the tanks and culture can be carried out *in situ*, with automatic temperature, pH and aeration control devices. Liquid sterilisation systems by filtration, for heat-labile components, can also be built into the fermenter. A 100 litre batch fermenter will yield a minimum of 66,000 doses (each of 3 ml) of OAV, and even more doses if the density is high enough for dilution to a reference standard equivalent to 1.5 g/litre, dry weight/volume.

OAV is made by the emulsification of equal volumes of a light mineral oil and the bacterial suspension, with 5% pure anhydrous lanolin as emulsifying agent. The mineral oil and lanolin are

first sterilised and, on cooling to 40°C, 0.5% formalin is added to the mixture. The bacterial suspension is added slowly and emulsification is continued for a further 10 minutes. Following overnight storage, the mixture is re-emulsified, bottled and stored at 4°C for 2 weeks prior to use.

APV is prepared by first adjusting the turbidity of the suspension to the reference standard as above, and diluting it with an equal volume of 0.5% formalinised physiological saline. The pH is adjusted to 6.5, and a hot 20% solution of potash alum is added to give a final concentration of 1% alum. After overnight storage with continuous agitation, the vaccine is bottled for use.

2.2.2. Requirements for substrates and media

A suitable sterilised medium for the aerated culture method is casein hydrolysate (2 g), sucrose (6 g), yeast extract (6 g), sodium chloride (5 g), anhydrous dipotassium hydrogen orthophosphate (8.6 g), anhydrous potassium dihydrogen orthophosphate (1.36 g), and distilled water to 1 litre. A denser growth is obtained if the casein, sucrose and yeast are prepared as a concentrate, filter-sterilised or autoclaved for 10 minutes at 107°C, and transferred aseptically into the tank that has previously been heat-sterilised with the rest of the ingredients.

2.2.3. In-process controls

Proper concentration of bacterial growth, the capsulation of the bacteria, purity of culture and efficient inactivation all need to be checked.

2.2.4. Final product batch tests

i) Sterility and purity

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

ii) Safety

Two seronegative cattle are vaccinated with twice the recommended dose and observed for 10–14 days for adverse effects.

Five mice are inoculated intramuscularly with 0.2 ml each of the vaccine, and observed for 5 days. The blood of any mouse that dies is cultured for *P. multocida*.

iii) Batch potency

Potency tests can be carried out by any of the following methods:

- a) Vaccination of cattle followed by direct challenge or passive mouse protection tests using the bovine sera. This procedure is not very feasible as cattle take a long time to develop adequate immunity after OAV;
- b) Vaccination of rabbits followed by direct challenge or passive mouse protection test using the rabbit sera; or
- c) Potency tests in mice, the most feasible method of the three.

Each of 50 mice is vaccinated intramuscularly with 0.2 ml of vaccine, and again 14 days later. On day 21, the mice are divided into ten groups of five, each group being challenged with respective dilutions of a 6–8-hour broth culture of a field strain in the range 10^{-1} – 10^{-10} ; 50 unvaccinated controls are similarly challenged, and all mice are observed for 5 days. The median lethal dose (LD₅₀) can then be calculated in order to obtain an indication of the dose that is sufficient to protect cattle: vaccines prepared in the manner described give at least 10^4 units protection in the vaccinated mice.

2.3. Requirements for regulatory approval

2.3.1. Safety requirements

i) Target and non-target animal safety

See chapter 1.1.8.

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

See chapter 1.1.8.

iii) Environmental consideration

See chapter 1.1.8.

2.3.2. Efficacy requirements

i) For animal production

A single dose of vaccine administered to young calves 4–6 months of age will protect susceptible animals for 3–4 months when APV is used, and for 6–9 months when OAV is used.

The vaccine should be administered by deep intramuscular injection. The use of nylon 5 ml volume syringes for a 3 ml dose and a gauge 14–15 needle is advised, and the recommended age for primary vaccination is 4–6 months. For routine, prophylactic vaccination, a single dose of OAV at 4–6 months, a booster 3–6 months later, and annual revaccination thereafter, is recommended. Where husbandry practices are such that reaching individual animals at appropriate times is impracticable, annual vaccination of all animals over 4 months of age, preferably before the breeding season, and vaccination of all calves under 1 year of age, 6 months later, is recommended. In the face of an outbreak in vaccinated animals, one dose of APV, followed by one dose of OAV, is recommended.

Leakage of OAV into subcutaneous tissue can occasionally give rise to fibrous lumps at sites of injection. Rarely, abscesses may develop if sterility conditions are not observed, though most animals are resistant to such infections. APV may occasionally cause shock reactions.

ii) For control and eradication

Not applicable.

2.3.3. Stability

The OAV emulsion should be pure white, and should stick to glass like paint. If the emulsion shows signs of cracking, it should be discarded. Separation of a thin layer of oil on the surface is permissible. It can be stored at 4–8°C for 6 months without any significant loss of potency. It must not be frozen. Increase in the content of lanolin improves stability, but also increases the viscosity – a distinct disadvantage. Use of other emulsifying agents such as 'Arlacel' helps to produce thinner, stable emulsions.

3. Vaccines based on biotechnology

Not applicable at present. However, several attempts are being made to develop defined live mutant vaccines, bacterial ghosts, as well as recombinant subunit vaccine with suitable adjuvants (Shivachandra *et al.*, 2011).

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* *

NB: At the time of publication (2025) there were no WOAHP Reference Laboratories for haemorrhagic septicaemia (please consult the WOAHP Web site:

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

NB: FIRST ADOPTED IN 1989; MOST RECENT UPDATES ADOPTED IN 2021.

Appendix 1.

Haemorrhagic septicaemia (*Pasteurella multocida* serotype 6:B and 6:E)

Intended purpose of test: population freedom from infection

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>No assays are available for this purpose. As <i>Pasteurella multocida</i> is known to be ubiquitous as well as commensal, it is difficult to use any assay to detect pathogenic organism and declare as freedom from potential threats.</u>							

Appendix 2.

Haemorrhagic septicaemia (*Pasteurella multocida* serotype 6:B and 6:E)

Intended purpose of test: individual animal freedom from infection prior to movement

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>The available assays have limited utility in declaring an animal as free from infection as <i>Pasteurella multocida</i> is known to be ubiquitous as well as commensal, it is difficult to use any assay to detect and differentiate pathogenic and non-pathogenic organisms and declare individual freedom from potential threats.</u>							

Appendix 3.

Haemorrhagic septicaemia (*Pasteurella multocida* serotype 6:B and 6:E)

Intended purpose of test: contribute to eradication policies

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>It is difficult to completely eradicate the pathogen from a population as it is multispecies and virulent.</u>							

Appendix 4.

Haemorrhagic septicaemia (*Pasteurella multocida* serotype 6:B and 6:E) Intended purpose of test: confirmation of clinical cases

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Bacterial isolation</u> <u>✓✓✓</u>	<u>Blood</u> <u>Nasal swab</u> <u>Tissues, bone marrow of long bones (femur, humerus etc.)</u>	<u>High accuracy</u>	<u>Use of samples from HS endemic and non-endemic areas</u>	<u>Standard Bacteriological methods</u>	<u>1. Pure culture possible directly from blood of infected live animal with high fever</u> <u>2. Pure culture possible directly from heart blood of infected dead animal</u>	<u>1. Non-availability of specific growth media</u> <u>2. Mixed cultures from Nasal swabs complicates isolation</u> <u>3. Isolation is possible from carrier/non-infected animal/healthy animals due to commensal nature of organism in nasopharynx/upper respiratory tract</u>	<u>Bergey's Manual for isolation of <i>Pasteurella multocida</i></u>
<u>Antigen detection</u> <u>✓✓✓</u>	<u>Blood/tissues</u>	<u>Not available</u>	<u>Use of samples from HS endemic and non-endemic areas</u>	<u>See reference</u>	<u>Easy to perform</u>	<u>Preparing standard antigen /antisera are laborious</u>	<u>Namioka (1978)</u>
<u>Mouse pathogenicity test^(a)</u> <u>✓✓✓</u>	<u>Pure culture / Mixed broth culture</u>	<u>Not available</u>	<u>Use of samples from HS endemic and non-endemic areas</u>	<u>As described</u>	<u>1. Aids in isolation of pure culture</u> <u>2. Results within 24–48 hours</u>	<u>1. Ethical issues</u> <u>2. Non-specific mortality some times</u> <u>3. Time consuming-2–3 days</u>	<u>≡</u>
<u>CIEP</u> <u>✓✓✓</u>	<u>Pure culture</u>	<u>Highly specific</u> <u>Low sensitivity</u>	<u>Use of samples from HS endemic and non-endemic areas</u>	<u>See reference</u>	<u>1. Specifically detects B and E cultures</u>	<u>1. Laborious</u> <u>2. Non-availability of standard sera</u> <u>3. Does not recognise other serotypes</u>	<u>Carter & Chengappa (1981)</u>
<u>Rapid slide agglutination test</u> <u>✓✓✓</u>	<u>Pure culture</u>	<u>Highly specific</u> <u>Low sensitivity</u>	<u>Use of samples from HS endemic and non-endemic areas</u>	<u>See reference</u>	<u>1. Simple and rapid test</u> <u>2. Results within 30 sec</u> <u>3. No other reagents required</u>	<u>1. Standard reference sera not available</u> <u>2. Non-availability of global reference laboratories</u>	<u>Heddleston <i>et al.</i> (1972)</u>
<u>PCR</u> <u>✓✓✓</u>	<u>Extracted DNA/ culture lysate</u>	<u>1. Highly specific to <i>P. multocida</i></u> <u>2. Highly sensitive assay DSe/DSp-not mentioned</u>	<u>Use of samples from HS endemic and non-endemic areas</u>	<u>See reference</u>	<u>1. Rapid identification of species and type</u> <u>2. Capsular and somatic typing</u>	<u>1. False-positive amplification of <i>P. canis</i> biotype 1</u> <u>2. HSB-PCR do not detect E:2 strains from Africa</u> <u>3. Needs equipment/expertise</u>	<u>Townsend <i>et al.</i> (1998; 2001)</u>

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Real-time PCR</u> ☒ ☒ ☒	<u>Extracted DNA/ culture lysate</u>	<u>1. Highly specific to <i>P. multocida</i></u> <u>2. Highly sensitive assay DSe/DSp-not mentioned</u>	<u>Use of samples from HS endemic and non-endemic areas</u>	<u>See reference</u>	<u>1. Rapid and real time identification /diagnosis</u> <u>2. More accurately identifies <i>P. multocida</i> isolates than PCR</u>	<u>1. Needs sophisticated equipment and expertise</u> <u>2. Expensive reagents</u>	<u>Petersen <i>et al.</i> (2014)</u>
<u>IHA</u> ☒ ☒ ☐	<u>Serum</u>	<u>1. Highly variable depending on type of antigen</u>	<u>Use of samples from HS endemic and non-endemic areas</u>	<u>See reference</u>	<u>1. Simple and relatively inexpensive assay</u> <u>2. Suitable for large-scale sero-surveys</u>	<u>1. Variable sensitivity, dependent on antigen quality, strain, and preparation</u> <u>2. Cross-reactions with other <i>Pasteurellae</i></u> <u>3. Performance differs in endemic vs non-endemic areas</u>	<u>Sawada <i>et al.</i> (1982)</u> <u>Sawada <i>et al.</i> (1985)</u>
<u>IHA</u> ☒ ☒ ☐	<u>Pure culture</u>	<u>1. Highly specific, sensitivity acceptable for typeable strains</u>	<u>Use of samples from HS endemic and non-endemic areas</u>	<u>See reference</u>	<u>1. Reliable serotyping method</u> <u>2. Useful for identification /selection of potential candidate strain</u>	<u>1. Requires type-specific antisera, which may not be available in all labs</u> <u>2. Laborious and less suited for routine diagnostic labs</u>	<u>Carter, 1955</u>

^(a)For animal welfare/ethical reasons, this test should only be used as a last resort.

Appendix 5.

Haemorrhagic septicaemia (*Pasteurella multocida* serotype 6:B and 6:E)

Intended purpose of test: prevalence of infection – surveillance

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
ELISA ☒ ☒ ☐	Serum	1. Highly variable depending on type of antigen/strain	Use of samples from HS endemic and non-endemic areas	See reference	1. Simple to assess the level of antibodies against the pathogen 2. Suitable for large-scale sero-surveys	1. Variable sensitivity, dependent on quality and type of antigen /strain used 2. Probability of cross-reacting with antibodies generated by other Pasteurellae	Tankaew <i>et al.</i> (2018) Shivachandra <i>et al.</i> (2011)
All the assays are suitable in limited circumstances.							

Appendix 6.

Haemorrhagic septicaemia (*Pasteurella multocida* serotype 6:B and 6:E)

Intended purpose of test: immune status in individual animals or populations post-vaccination

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
At present, there is no globally accepted standard immune assay except IHA to monitor antibodies status. However, repeatability of IHA had shown a serious concern due to variability in antigen preparation. Similarly, ELISA has been used by some researchers with limited success in monitoring, post-vaccination antibodies/immune status in animals. ELISA performed using whole cell (heat killed/sonicated/ boiled) antigen or OMPs/IROMPs with lab-to-lab variation in antigen preparation found to provide variable reactivity. Cross-reactivity is possible with immunity raised against other capsular/somatic serotypes.							

MEETING OF THE BIOLOGICAL STANDARDS COMMISSION

Paris, 8–12 September 2025

CHAPTER 3.4.11.

**INFECTIOUS BOVINE RHINOTRACHEITIS/
INFECTIOUS PUSTULAR VULVOVAGINITIS**

SUMMARY

Description of disease: Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis (IBR/IPV), caused by bovine alphaherpesvirus 1 (BoAHV-1) ~~of the species~~ Varicellovirus bovinealpha 1, is a disease of domestic and wild cattle. The virus is distributed world-wide but has been eradicated from a number of European countries and others have active eradication programmes.

The disease is characterised by clinical signs of the upper respiratory tract, such as a (muco)purulent nasal discharge, hyperaemia of the muzzle (red nose disease) and by conjunctivitis. Signs of general illness are fever, depression, inappetence, abortions and reduced milk yield. The virus can also infect the genital tract and cause pustular vulvovaginitis and balanoposthitis. Post-mortem examinations reveal rhinitis, laryngitis and tracheitis. Mortality is low, and most infections run a subclinical course. Secondary bacterial infections can lead to more severe respiratory disease, and BoAHV-1 could play a role in multifactor diseases such as 'shipping fever'.

Identification of the agent: The virus can be isolated from nasal or genital swabs from animals with respiratory signs, vulvovaginitis or balanoposthitis, taken during the acute phase of the infection, and, in severe cases, from various organs collected at post-mortem. Following infection, BoAHV-1 may persist in infected animals in a latent state in sensory neurons, e.g. in the trigeminal or sacral ganglia. The virus can be reactivated, and this results in virus shedding (re-excretion) without exhibition of clinical disease. Because of this latency phenomenon, antibody-positive animals have to be classified as infected with BoAHV-1 (with two exceptions: serological responses induced by vaccination with an inactivated vaccine or by colostral antibodies).

For virus isolation, various cell cultures of bovine origin are used, for example, secondary lung or kidney cells or the Madin–Darby bovine kidney cell line. The virus produces a cytopathic effect in 2–4 days. It is identified by neutralisation ~~or~~ antigen detection methods using monospecific antisera or monoclonal antibodies or by genome detection. BoAHV-1 isolates can be further subtyped by DNA restriction enzyme analysis into subtypes 1.1 and 1.2. BoAHV-1.2 isolates can be further differentiated into 2a and 2b. Development of rhinotracheitis or vulvovaginitis/balanoposthitis depends more on the route of infection than on the subtype of the virus. The virus previously referred to as BoAHV-1.3, a neuropathogenic agent, is now classified as BoAHV-5.

For virus DNA detection, the polymerase chain reaction (PCR) technique is increasingly used in routine diagnosis, especially the real-time PCR.

Serological tests: The virus neutralisation test and various enzyme-linked immunosorbent assays (ELISA; indirect or blocking) are most widely used for antibody detection. With the ELISAs, antibodies can be detected in serum or plasma, and with ~~lower sensitivity~~ specialised indirect milk ELISAs also

in milk or bulk milk samples. The use of a gE-antibody-ELISA makes it possible to distinguish field virus infected cattle from cattle vaccinated with a gE-deleted marker vaccine (DIVA strategy).

Requirements for vaccines: Inactivated and attenuated live vaccines are available. The vaccines protect cattle clinically in case of infection and markedly reduce the subsequent shedding of field virus. Although vaccination may not prevent field virus infection of individual animals, spreading of wild-type virus in infected herds is efficiently reduced. The vaccines must not induce disease, abortion, or any local or systemic reaction, and must be genetically stable. BoAHV-1 glycoprotein E deleted mutant marker vaccines are now generally available (live or inactivated) and can be used as part of a DIVA strategy.

A. INTRODUCTION

Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis (IBR/IPV) is caused by viruses of the species *Varicellovirus bovinealpha 1* (Gatherer et al., 2021). The bovine *alphaherpesviruses of type 1* (BoAHV-1), is a, IBR-virus) of this virus species induce disease of domestic and wild cattle. BoAHV-1 is a member of the genus *Varicellovirus* in the subfamily *Alphaherpesvirinae*, which belongs to the *Orthoherpesviridae* family, order *Herpesvirales*. The viral genome consists of a double-stranded DNA with a size of approximately 135,000 base pairs that encodes for about 70 proteins, of which 33 structural and more than 15 nonstructural proteins have been identified. The viral glycoproteins, which are located in the envelope on the surface of the virion, play an important role in pathogenesis and immunity. BoAHV-1 can be further differentiated into subtypes 1.1, 1.2a and 1.2b (Metzler et al., 1985). The BoAHV-1.2 subtypes may be less virulent than subtype 1.1 (Edwards et al., 1990). The former BoAHV-1.3, which may act as a neuropathogenic agent in calves, has been re-classified as BoAHV-5 (Magyar et al., 1993). BoAHV-1 shares close antigenic and genetic close relationships with other ruminant alphaherpesviruses: BoAHV-5, caprine alphaherpesvirus 1, cervid alphaherpesvirus 1 (red deer), cervid alphaherpesvirus 2 (reindeer), bubaline alphaherpesvirus 1 and elk alphaherpesvirus 1 (Thiry et al., 2006).

After an incubation period of 2–4 days, serous nasal discharge, salivation, fever, inappetence, and depression become evident. Within a few days the nasal and ocular discharges change to mucopurulent. Where natural mating is practised, genital infection can lead to pustular vulvovaginitis or balanoposthitis. However, most infections run a very mild or subclinical course (Van Oirschot et al., 1993). Uncomplicated cases of respiratory or genital disease caused by BoAHV-1 last about 5–10 days. Secondary bacterial or viral agents may contribute to a multifactor disease complex resulting in severe respiratory disease of young animals ('shipping' or 'crowding fever').

After infection via the airborne route, BoAHV-1 replicates to high titres in mucous membranes of the upper respiratory tract and in the tonsils. Subsequently, the virus disseminates to conjunctivae and reaches the trigeminal ganglia by neuronal axonal transport. After genital infection, BoAHV-1 replicates in the mucous membranes of the vagina or prepuce and becomes latent in the sacral ganglia. The viral DNA remains in the neurons of the ganglia, probably for the entire life of the host (status of latency). Stress, such as transport, regrouping, other diseases and parturition, but also the application of corticosteroids can induce reactivation of the latent infection. Consequently, the virus may switch between latent and lytic infection and may be shed intermittently into the environment and spread to contact animals ~~shed intermittently into the environment and spread to contact animals~~. The virus is relatively resistant to environmental influences but only at low temperatures in a humid atmosphere. Inactivation of BoAHV-1 in the environment depends on temperature, pH, light, humidity, and the matrix harbouring the virus. Virus infectivity is stable for up to 1 month at 4°C. At higher temperatures the virus is inactivated more rapidly: 6 log₁₀ TCID₅₀ (50% tissue culture infective doses) per ml of BoAHV-1 were inactivated, under laboratory conditions, within 21 minutes at 56°C, 10 days at 37°C, 50 days at 22°C and within 14 days under field conditions (Gibbs & Rweyemamu 1977; Snowdon, 1965). BoAHV-1 can survive for 30 days during cold periods when the relative humidity is above 90%. In warmer environments, such as in buildings, survival between 5 and 13 days has been recorded. In favourable conditions, e.g. in feed stuffs, the virus may survive for more than 30 days (Wentink et al., 1993). The virus is rapidly and completely inactivated by a broad spectrum of commercially available disinfectants.

BoAHV-1 infection elicits an antibody response and a cell-mediated immune response within 7–14 days. The immune response is presumed to persist life-long, although it may fall below the detection limit of some tests after a number of years. Maternal antibodies are transferred via colostrum to the young-newborn calf, which is consequently protected against BoAHV-1-induced clinical disease but only to a very limited extend against establishment of an infection (Mechor et al., 1987). Maternal antibodies have a biological half-life of about 3 weeks, but may and can be detected occasionally in animals for several up to 6–9 months.

The virus is distributed worldwide, with the exception of the BoAHV-1-free countries, paralleling the distribution of domestic cattle. Other *Artiodactyla* (e.g. goats, sheep, water buffaloes, camelids) may be infected with BoAHV-1. After infection of cattle, nasal viral shedding is detected for 5–14 days, with peak titres of 10⁸–10¹⁰ TCID₅₀ per ml of nasal secretion. The highest virus titre is shed between days 4 and 6 post-infection. The semen of an infected bull

may contain BoAHV-1 for several weeks, and the virus can thus be transmitted by natural mating and artificial insemination (Parsonson & Snowdon, 1975).

Vaccines usually prevent the development of clinical signs and markedly reduce the shedding of virus after infection, but do not completely prevent infection and even establishment of latency. Several eradication campaigns have been carried out or are currently running in different countries including test-and-removal programmes or vaccination campaigns (see Section C).

BoAHV-1 infection may be suspected on the basis of clinical, pathological and epidemiological findings. However, to make a definite diagnosis, laboratory ~~examinations (serology or investigations such as virus detection)~~ or serology are required. A complete diagnostic procedure in the laboratory is aimed at detecting the causative virus (or viral components) and the specific antibodies they induce. Nevertheless, because of latent infection induced by BoAHV-1, detection of antibodies ~~could be~~ is sufficient for the determination of the BoAHV-1 status of individual animals except for animals that are vaccinated with conventional BoAHV-1 vaccines and for young animals with colostral antibodies.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of BoAHV-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,h)						
Virus isolation	□□□	☑□□ ^(d-g,h)	☑□□	☑☑□	□□□-☑□□	□□□
Real-time PCR	□□□	☑□□-☑☑□ ^(d-g,h)	☑□□	☑☑☑	□□□-☑□□	□□□
Detection of immune response						
ELISA	☑☑☑	☑☑☑	☑☑☑	☑☑□	☑☑☑	☑☑☑ ⁽ⁱ⁾
VN	☑☑□	☑☑□	☑☑□	☑□□	☑☑□	☑☑□ ⁽ⁱ⁾

Key: ☑☑☑ = recommended for this purpose; ☑☑□ = recommended but has limitations;

☑□□ = suitable in very limited circumstances; □□□ = not appropriate for this purpose.

PCR = polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay; VN = virus neutralisation.

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

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

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

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

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

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

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

^(g-h)Virus detection is not suitable to determine individual animal freedom in the latent stage of the infection.

^(h)Method particularly applicable to testing of semen.

⁽ⁱ⁾Limited to marker vaccination.

1. Identification of the agent

1.1. Collection and processing of specimens

Nasal swabs, preferably foam or flocked swabs, are collected from several (from five to ten) affected cattle in the early phase of the infection (feverish and antibody negative). These cattle still have serous rather than mucopurulent nasal discharge. In cases of vulvovaginitis or balanoposthitis, swabs are taken from the genitals. The swabs should be vigorously rubbed against the mucosal surfaces. The prepuce can also be washed with saline; the washing fluid is then collected. The specimens are suspended in transport medium (cell culture medium containing antibiotics and 2–10% BoAHV-1-free fetal bovine serum to protect the virus from inactivation), ~~cooled~~ chilled at 4°C, and rapidly submitted to the laboratory.

During necropsy, mucous membranes of the respiratory tract, and samples of the tonsil, lung and bronchial lymph nodes are collected for virus detection. In cases of abortion, the fetal liver, lung, spleen, kidney and placental cotyledons are examined. Samples should be kept on ice and sent to the laboratory as quickly as possible.

After arrival at the laboratory, swabs are agitated at room temperature for 30 minutes in the transport medium to elute the virus. Following removal of the swabs, the transport medium is clarified by centrifugation at 1500 *g* for 10 minutes. Tissues are homogenised to a 10–20% (w/v) suspension in cell culture medium before centrifugation at 1500 *g* for 10 minutes. The supernatants of these specimens are filtered through 0.45–22 μ m filters and used for virus isolation.

~~The~~ Isolation of the virus from semen needs some special adaptations, because the seminal fluid contains enzymes and other ~~factors~~ components that are toxic to the cells and inhibit viral replication (see below).

1.2. Virus isolation

For virus isolation, bovine cells of various origins can be used. Primary or secondary bovine kidney, lung or testis cells, cell strains derived from bovine fetal lung, turbinate or trachea, and established cell lines, such as the Madin–Darby bovine kidney cell line (MDBK), are suitable for BoAHV-1 propagation. Cells ~~cultures~~ can be grown in glass or plastic tubes, plates or dishes. When 24-well plastic plates are used, a 100–200 μ l volume of the supernatants described above is inoculated into these cell cultures. After a 1-hour adsorption period, the cultures are rinsed ~~and with~~ maintenance medium ~~is added containing~~ antibiotics and 2% serum (preferably fetal calf serum [FCS]). The serum used as a medium supplement in the maintenance medium should be free of antibodies against BoAHV-1. The cell cultures are observed daily for cytopathic effect (CPE) (see Figure 1), which usually appears within 1–3 days after inoculation. It is characterised by grape-like clusters of rounded cells gathered around a hole in the monolayer; sometimes giant cells with several nuclei may be observed. Experience is needed to recognise this characteristic appearance. ~~When, after 7 days, If no CPE has appeared after 7 days,~~ a blind passage must be made. The cell culture is freeze–thawed and clarified by centrifugation, and the supernatant is used for inoculation of fresh monolayers (Brunner *et al.*, 1988; ~~Edwards et al., 1983~~).

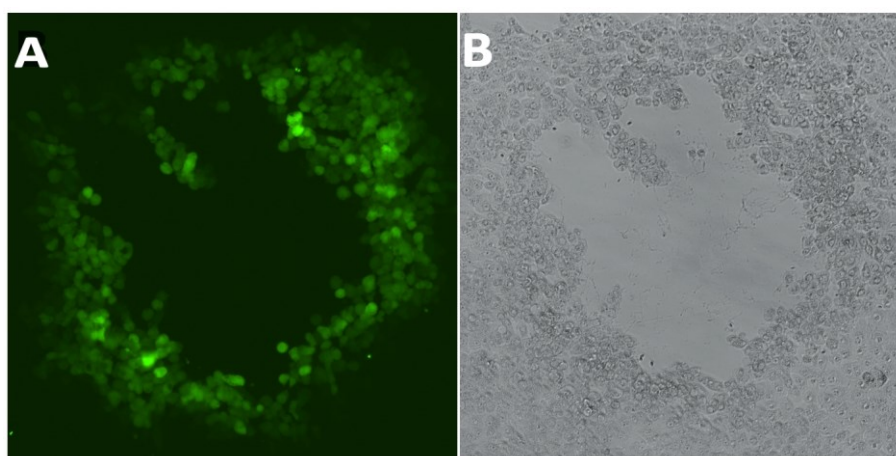


Fig. 1. BoAHV-1 CPE. A) Autofluorescence of a green fluorescent protein (GFT) expressing BoAHV-1 on Madin–Darby bovine kidney (MDBK) cultures with typical plaque formation and rounded cells in grape shaped clusters. B) Corresponding bright-field image.

To verify the infectious agent as BoAHV-1, cell culture samples or aliquots of the supernatants are analysed by PCR test (see below, Section B.1.3 *Molecular methods – detection of nucleic acids*). An alternative method of virus identification is the direct verification of BoAHV-1 antigen in cells around the CPE by an immunofluorescence or immunoperoxidase test with conjugated monospecific antiserum or monoclonal antibody (MAb) (commercially available). Furthermore, the supernatant can be used as template for restriction endonuclease fragment length polymorphism (RFLP) (see Section B.1.5 *Differentiation of bovine herpesvirus 1 subtypes and of ruminant alphaherpesviruses related to bovine herpesvirus 1*). To identify the recovered virus as BoAHV-1, the supernatant of the culture ~~should can~~ also be ~~neutralised~~ used in virus neutralisation test (VNT) with a monospecific BoAHV-1 antiserum or neutralising monoclonal antibody (MAb). For this purpose, serial tenfold dilutions of the test supernatant are made, and to each dilution monospecific BoAHV-1 antiserum or negative control serum is added. Following incubation at 37°C for 1 hour, the mixtures are inoculated into cell cultures; 3–5 days later, the neutralisation index is calculated. The neutralisation index is the virus titre (in log₁₀) in the presence of

negative control serum minus the virus titre in the presence of specific antiserum. If the neutralisation index is greater than 1.5, the isolate may be considered to be BoAHV-1. To shorten the virus isolation procedure, two specimens may be inoculated into cell culture: one that has been pre-incubated with monospecific antiserum and another that has been preincubated with negative control serum. If the CPE is inhibited by the monospecific antiserum, the isolate can be considered to be BoAHV-1, although definitive confirmation would require molecular characterisation to distinguish it from related ruminant alphaherpesviruses.

~~An alternative method of virus identification is the direct verification of BoHV-1 antigen in cells around the CPE by an immunofluorescence or immunoperoxidase test (Kaashoek *et al.*, 1994) with conjugated monospecific antiserum or MAb. Furthermore, the supernatant can be used as template for restriction endonuclease fragment length polymorphism (RFLP) (see Section B.1.4) and polymerase chain reaction (PCR) analyses (see Section B.1.3).~~

1.2.1. Virus isolation from semen

0.05 to 0.1 ml of raw semen should be tested with two passages in cell culture. Raw semen is generally cytotoxic and should be prediluted (e.g. 1/10) before being added to cell cultures. A similar problem may sometimes arise 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 0.5 ml extended semen corresponding to one straw). Multiple diluted samples may need to be tested with this procedure to reach a volume equivalent to 0.1 ml raw semen (e.g. ~~5~~ 1 ml of a 1/10 diluted sample of extended semen). ~~A suitable test~~ The detail of the procedure is given below. At least three different straws per batch of extended semen should be analysed in duplicates each. See also Brunner *et al.* (1988).

1.2.1.1. Test procedure

- i) Dilute 200 µl fresh semen in 2 ml fetal bovine serum (free from antibodies against BoAHV-1) with antibiotics; carry out the same procedure with 1/10 and 1/100 dilutions of the ejaculate or 1:10 dilutions of the corresponding equivalent of extended semen.
- 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 above) in a six-well tissue culture plate.
- iv) Incubate the plates for 1 hour at 37°C.
- v) Remove the mixture, wash the monolayer twice with 5 ml maintenance medium, and add 5 ml maintenance medium to each well.
- vi) Include BoAHV-1 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 control last, and using separate plates.
- vii) Observe plates under a microscope daily for the appearance of a CPE. If a CPE appears, confirmatory tests for BoAHV-1 are made by PCR, immunolabelling methods or specific neutralisation or immunolabelling methods (see above).
- viii) If there is no CPE after 7 days, the cultures are frozen and thawed, clarified by centrifugation, and the supernatant is used to inoculate fresh monolayers.
- ix) The sample is considered ~~to be as~~ negative, if there is no evidence of a CPE after 7 days' incubation of the passaged cultures.

1.3. Molecular methods – detection of nucleic acids ~~Nucleic acid detection~~

1.3.1. Extraction of nucleic acids

Different kits and procedures can be used for nucleic acid extraction. The recommended methods for BoAHV-1 genome detection are DNA isolation after Proteinase K digestion (spin column, ~~Chelex chelating~~ 100 resin) or accordingly magnetic bead based nucleic acid extraction. The quality and concentration of the extracted nucleic acid is critical and must be validated using a suitable method as appropriate to the circumstances including internal controls for PCR assays.

1.3.1.1. Sample preparation, equipment and reagents

- i) The samples used for the test are, typically, extended bovine semen stored in liquid nitrogen. The semen samples can be transported to the laboratory in liquid nitrogen, or shipped at 4°C, and stored at 4°C (for short-term storage) or in liquid nitrogen or at -70°C (for long-

term storage) or 4°C (for short term storage). Storing semen at 4°C for a short period (up to 7 days) does not affect PCR test results.

- ii) Three straws from each batch of semen should be processed. Duplicate PCR amplifications should be carried out for each DNA preparation (six amplifications in total) to ensure the detection of DNA in samples containing low levels of virus.
- iii) The real-time PCR assay described here involves two separate procedures. Firstly, BoAHV-1 DNA is extracted from semen using chelating 100 resin (styrene divinylbenzene copolymer [100 mesh]), along with proteinase K and DL-dithiothreitol (DTT). The second procedure is the PCR analysis of the extracted DNA template in a real-time PCR reaction mixture. A number of commercial real-time PCR amplification kits are available, and any particular kit selected needs to be compatible with the chosen real-time PCR platform. The required primers and probes can be synthesised by various commercial companies, but all should be HPLC purified.

1.3.1.2. Extraction of DNA

- i) In a screw top 1.5 ml tube, add:

Chelating 100 resin sodium (10% w/v in distilled deionised water)	100 µl
Proteinase K (10 mg/ml)	11.5 µl
DL-dithiothreitol (1 M)	7.5 µl
Nuclease-free water	90 µl
Semen sample	10 µl

Mix gently by pipetting¹.

- ii) The samples are incubated at 56°C for 30 minutes and then vortexed at high speed for 10 seconds.
- iii) Subsequently, the tubes are incubated in a boiling water bath for 8 minutes and then vortexed at high speed for 10 seconds.
- iv) The tubes are centrifuged at 10,000 g for 3 minutes.
- v) The supernatant² is transferred into a new microtube and can be used directly for PCR, or stored at -20°C.

1.3.2. PCR

During the past decade, various methods for the detection of BoAHV-1 DNA in clinical samples have been described, including DNA-DNA hybridisation and PCR. PCR is increasingly being used in routine diagnostic submissions (Moore *et al.*, 2000). Compared with virus isolation, PCR has the primary advantages of being more sensitive and ~~more~~ rapid: it can be performed in 1–2 days. It is also possible to detect episomal DNA of non-replicating virus in sensory ganglia (Van Engelenburg *et al.*, 1993), such as the trigeminal ganglion, in the latent phase of infection when virus isolation is not successful due to the absence of virus in an infectious state. The disadvantage is that PCR ~~analyses~~ procedures are prone to contamination and therefore precautions have to be taken to prevent false-positive results. Risk of contamination is markedly reduced by new PCR techniques, such as real-time quantitative PCR (see below) (Abril *et al.*, 2004; Lovato *et al.*, 2003; Wernike *et al.*, 2011) and the PCR method is recommended in all cases for direct BoAHV-1 detection.

So far, PCR has been used mainly to detect BoAHV-1 DNA in artificially (Kramps *et al.*, 1993) or naturally (Van Engelenburg *et al.*, 1993) infected semen samples, however, it can also be used for disease investigation where IBR is clinically suspected. It is important to thoroughly optimise the PCR conditions, including the preparation of the samples, the concentration of test components e.g. primers and polymerase, and the cycle programmes. The target region for amplification must be present in all BoAHV-1 strains, and its nucleotide sequence for the primer- and probe-binding sites must be conserved. The TK, gB, gC, gD and gE genes have been used as targets for PCR amplification. In addition, PCRs based on detection of gE sequences can be used to differentiate between wild-type virus and gE-deleted vaccine strains (Fuchs *et al.*, 1999; Schyns *et al.*, 1999; Wernike *et al.*, 2011). Discrimination between infection with virulent IBR strains and infection with live attenuated (non-marker) vaccine strains may not be easily achievable with the PCR technique, and RFLP or high throughput sequencing (HTS) can be used for this purpose. Specific PCRs have been developed that are able to discriminate between

- 1 It is important that the Chelex-chelating 100 resin solution is homogeneous while pipetting, as Chelex-chelating 100 resin sodium is not soluble. This can be achieved by putting the vessel containing Chelex-100 the solution on a magnetic stirrer while pipetting.
- 2 Some DNA samples can become cloudy, and a thin white membrane may form occasionally after freezing and thawing. This appears to have no influence on the PCR performance. No heating or re-centrifuging of the samples is necessary.

BoA~~H~~V-1, BoA~~H~~V-5 and other related ~~alphaherpesviruses~~ varicelloviruses (Ashbaugh *et al.*, 1997; Ros *et al.*, 1999; Wernike *et al.*, 2021).

Experimentally, PCR was found to be more sensitive than virus isolation: in egg yolk-extended semen samples obtained from experimentally infected bulls, PCR detected five times as many positives as did virus isolation (Van Engelenburg *et al.*, 1995). The detection limit of validated PCR assays amounts to only a few genome copies per PCR-reaction. Nevertheless, false-negative results cannot be excluded. To identify possible false-negative results, it is recommended to spike an internal control template into the reaction tube of the semen sample to be amplified by the same primers. Such a control template may be constructed by inserting, for example, a 100 base-pair fragment into the target region. This control template also makes it possible to semi-quantify the amount of DNA that is detected (Ros *et al.*, 1999; Van Engelenburg *et al.*, 1993). When using an internal control, extensive testing is necessary to ensure that PCR amplification of the added internal control does not compete with the diagnostic PCR and thus lower the analytical sensitivity (see also Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*). DNA extraction and quality of the DNA preparations as well as presence of PCR inhibitors in the diagnostic sample can also be controlled by amplification of cellular sequences (housekeeping genes, Wernike *et al.*, 2011) or by addition of 'artificial' DNA sequences prior to extraction procedures (e.g. green fluorescent protein gene, non-BoHV-related viruses) as internal controls. To enhance the sensitivity and specificity of the BoA~~H~~V-1 PCR, real-time PCR systems are the methods of choice.

~~1.3.1. Real time polymerase chain reaction~~

The following real-time PCR test methods ~~has~~ have been developed to detect BoA~~H~~V-1 in extended bovine semen destined for trade. The methods ~~has~~ have been validated according to chapter 1.1.6, and includes a comprehensive international inter-laboratory comparison involving six collaborating laboratories with specialist status in IBR testing (Wang *et al.*, 2008; Wernike *et al.*, 2011).

A number of studies have shown that PCR assays are more sensitive than virus isolation, especially in a late stage of acute infection with decreasing viral loads and presence of neutralising antibodies (Smits *et al.*, 2000; Van Engelenburg *et al.*, 1995; Vilcek *et al.*, 1994; Wang *et al.*, 2008; Wiedmann *et al.*, 1993). Real time PCR has been used for the detection of BoHV-1 and BoHV-5 in experimentally infected cattle and mice (Abril *et al.*, 2004; Lovato *et al.*, 2003) and A series of conventional PCR assays have been used for the detection of BoA~~H~~V-1 DNA in artificially or naturally infected bovine semen samples (Deka *et al.*, 2005; Grom *et al.*, 2006; Masri *et al.*, 1996; Van Engelenburg *et al.*, 1993; Weiblen *et al.*, 1992; Wiedmann *et al.*, 1993; Xia *et al.*, 1995). Conventional detection of amplified PCR products relies on gel electrophoresis analysis ~~(Rola *et al.*, 2003)~~. Sequence-specific primers have been selected to amplify different parts of conserved glycoprotein genes of the BoA~~H~~V-1 genome, including glycoprotein B (gB) gene (Abril *et al.*, 2004; Grom *et al.*, 2006; Santurde *et al.*, 1996), gC gene (Smits *et al.*, 2000; Van Engelenburg *et al.*, 1995), gD gene (Smits *et al.*, 2000; Wiedmann *et al.*, 1993), gE gene (Grom *et al.*, 2006), and the thymidine kinase (tk) gene (Moore *et al.*, 2000; Yason *et al.*, 1995).

Real-time PCR has been used for the detection of BoA~~H~~V-1 and BoA~~H~~V-5 in experimentally infected cattle and mice (Abril *et al.*, 2004). Furthermore, a triplex real-time PCR for the detection of BoA~~H~~V-1 gD and gE genes together with a house-keeping gene was validated and reported in 2011 (Wernike *et al.*, 2011). This PCR also allows the easy and fast differentiation of BoA~~H~~V-1 field strains from gE-deleted vaccine strains. The real-time PCR~~s~~ described here uses a pair of sequence-specific primers for amplification of target DNA and a 5'-nuclease oligoprobe for detection of amplified products. The oligoprobe is a single, sequence-specific oligonucleotide, labelled with two different fluorophores, ~~the reporter/donor 5-carboxyfluorescein (FAM) a "fluorescent dye" at the 5'-one end,~~ and ~~the acceptor/ a "dark fluorophore" (quencher-6-carboxytetramethylrhodamine (TAMRA) at the 3'-other end.~~ This/These real-time PCR assays is are designed to detect viral DNA of all BoA~~H~~V-1 strains, including subtype 1 and 2, from extended bovine semen and other clinical samples such as nasal swabs taken from IBR suspected animals. The assay selectively amplifies a 97 base-pair sequence of the glycoprotein B (gB) gene. Details of the primers and probes are given in the protocol outlined below. The method by Abril *et al.* (2004) also detects BoA~~H~~V-5, bubaline alphaherpesvirus 1, cervid alphaherpesvirus 1 and caprine alphaherpesvirus 1, therefore a positive outcome may require additional differentials.

~~1.3.2.1. Sample preparation, equipment and reagents~~

- i) ~~The samples used for the test are, typically, extended bovine semen stored in liquid nitrogen. The semen samples can be transported to the laboratory in liquid nitrogen, or shipped at 4°C, and stored at 4°C (for short term storage) or in liquid nitrogen or at -70°C (for long~~

term storage) or 4°C (for short term storage). Storing semen at 4°C for a short period (up to 7 days) does not affect PCR test result.

- ii) ~~Three straws from each batch of semen should be processed. Duplicate PCR amplifications should be carried out for each DNA preparation (six amplifications in total) to ensure the detection of DNA in samples containing low levels of virus.~~
- iii) ~~The real time PCR assay described here involves two separate procedures. Firstly, BoA¹HV-1 DNA is extracted from semen using Chelex-100 chelating resin, along with proteinase K and DL-dithiothreitol (DTT). The second procedure is the PCR analysis of the extracted DNA template in a real-time PCR reaction mixture. A number of commercial real-time PCR amplification kits are available, and any particular kit selected needs to be compatible with the chosen real-time PCR platform. The required primers and probes can be synthesised by various commercial companies, but all should be HPLC purified.~~

1.3.2.2. Extraction of DNA

- i) In a screw top 1.5 ml tube, add:

Chelex 100 sodium (10% w/v in distilled deionised water)	100 µl
Proteinase K (10 mg/ml)	11.5 µl
DL-dithiothreitol (1 M)	7.5 µl
Nuclease free water	90 µl
Semen sample	10 µl

Mix gently by pipetting³.

- ii) ~~The samples are incubated at 56°C for 30 minutes and then vortexed at high speed for 10 seconds.~~
- iii) ~~Subsequently, the tubes are incubated in a boiling water bath for 8 minutes and then vortexed at high speed for 10 seconds.~~
- iv) ~~The tubes are centrifuged at 10,000 g for 3 minutes.~~
- v) ~~The supernatant⁴ is transferred into a new microtube and can be used directly for PCR, or stored at -20°C.~~

1.3.2.1.4.3. Preparation of reagents

The manufacturer's instructions should be followed for application and storage of the real-time PCR reaction mixtures.

Working stock solutions for primers and probes are made with nuclease-free water at the concentration of 4.5 µM and 3 µM, respectively in suitable concentrations. The stock solutions are stored at -20°C and the probe solution should be kept in the dark protected from light. Single-use aliquots can be prepared to limit freeze-thawing of primers and probes and extend their shelf life.

1.3.2.2.4.4. Real-time PCR test procedure

- i) *Primers and probe sequences*

Selection of the primers and probe are outlined in Abril *et al.* (2004) and described below.

Primer gB-F: 5'-TGT-GGA-CCT-AAA-CCT-CAC-GGT-3' (position 57499-57519 GenBank®, accession AJ004801)

Primer gB-R: 5'-GTA-GTC-GAG-CAG-ACC-CGT-GTC-3' (position 57595-57575 GenBank®, accession AJ004801)

Labelled Probe: 5'-FAM-AGG-ACC-GCG-AGT-TCT-TGC-CGC-TAMRA-3' (position 57525-57545 GenBank®, accession AJ004801)

Target gene	Primer/probe (5'-3')	Final concentration	Cycling parameters ^(a)
Method 1: Abril <i>et al.</i> (2004); GenBank Accession No.: AJ004801, amplicon size: 97 bp			

³ It is important that the Chelex 100 solution is homogeneous while pipetting, as Chelex 100 sodium is not soluble. This can be achieved by putting the vessel containing Chelex-100 solution on a magnetic stirrer while pipetting.

⁴ Some DNA samples can become cloudy, and a thin white membrane may form occasionally after freezing and thawing. This appears to have no influence on the PCR performance. No heating or re-centrifuging of the samples is necessary.

gB	Primer gB-F: TGT-GGA-CCT-AAA-CCT-CAC-GGT Primer gB-R: GTA-GTC-GAG-CAG-ACC-CGT-GTC Labelled Probe: FAM-AGG-ACC-GCG-AGT-TCT-TGC-CGC-TAMRA	0.24 µM 0.24 µM 0.16 µM	2 min 50°C, 10 min 95°C 45 cycles of ^(a) : 95°C/15 sec and 60°C/45 sec
Method 2: Wernike <i>et al.</i> (2011); GenBank Accession No.: AJ004801, amplicon size: gD 109 bp, gE 106 bp, β-actin 105 bp			
gD	BoAHV-1-gD5595-F: CCG-CCG-TAT-TTT-GAG-GAG-TCG BoAHV-1-gD5595-R: TCG-GTC-TCC-CCT-TCR-TCC-TC BoAHV-1-gD-TEX: Texas Red-TAC-GAG-CCG-CCG-CCT-GCC-GC-BHQ2	0.8 µM 0.8 µM 0.1 µM	15 min 95°C 42 cycles of ^(a) : 95°C/60 sec, 58°C for 30 sec and 72°C/30 sec
gE	BoAHV-1-gE3-883-F: CAA-TAA-CAG-CGT-AGA-CCT-GGT-C BoAHV-1-gE3-989-R: GCT-GTA-GTC-CCA-AGC-TTC-CAC BoAHV-1-gE3-FAM: FAM-TGC-GGC-CTC-CGG-GCT-TTA-CGT-CT-BHQ1	0.8 µM 0.8 µM 0.15 µM	
β-actin	ACT-1030-F: AGC-GCA-AGT-ACT-CCG-TGT-G ACT-1135-R: CGG-ACT-CAT-CGT-ACT-CCT-GCT-T ACT-1081-HEX: HEX-TCG-CTG-TCC-ACC-TTC-CAG-CAG-ATG-T-BHQ1	0.2 µM 0.2 µM 0.1 µM	

^(a)PCR parameters may vary with different PCR platforms.

ii) *Preparation of reaction mixtures*

The PCR ~~reaction~~ mixtures are prepared in a separate laboratory room. For each PCR test, appropriate controls should be included. As a minimum, a no template control (NTC; ~~reagents only~~), appropriate negative controls, i.e. 1 per 10 test samples, and two positive controls (moderate and weak positive) should be included. Each test sample and control ~~is~~ are tested in duplicate. The PCR amplifications are carried out in a volume of 25 µl.

- a) PCR reagent mixtures (nuclease free water, reaction buffer, primers and probes and given case reference dyes) are added in a clean room (no viral cultures, DNA extracts or post-amplification products should be handled here) to prepare a master mix.

2 × real time PCR reaction mixture	12.5 µl
ROX reference dye (optional)	0.5 µl
Forward primer (gB-F, 4.5 µM)	1 µl
Reverse primer (gB-R, 4.5 µM)	1 µl
Probe (3 µM)	1 µl
Nuclease free water	4 µl

- b) 5 µl of the DNA template are added to the PCR reagent mixture to a final volume of 25 µl. DNA samples are prepared in a separate room and added to the PCR mix in a separate room or under a PCR working station with UV decontamination facility.

iii) Real-time polymerase chain reaction

The PCR tubes are placed in the real-time PCR detection system in a separate, designated PCR room. The PCR detection system is programmed for the test as ~~follows: above.~~

~~PCR Reaction Parameters (may vary with different PCR platforms)~~

~~One cycle:~~	~~Hold 50°C~~	~~2 minutes~~
~~One cycle:~~	~~Hold 95°C~~	~~2 minutes~~
~~45 cycles:~~	~~Hold 95°C~~	~~15 seconds~~
	~~Hold 60°C~~	~~45 seconds~~

iv) Analysis of real-time PCR data

The threshold level is usually set according to the manufacturers' instructions for the selected analysis software used. Alternatively, virus isolation negative semen samples, from seronegative animals, can be run alongside to determine the background signal associated with the detection system used.

1.3.2.3.4.5. Interpretation of results

i) Test controls

Positive and negative controls, as well as reagent controls, should be included in each PCR test. Negative semen, ~~from seronegative bulls,~~ tissue, swab or cell culture can be used as

a negative processing control matching the samples in-test. Positive semen from naturally infected bulls is preferable as a positive extraction control if semen samples are being tested. However, this might be difficult to obtain. Alternatively, positive extraction controls can be derived from negative semen spiked with defined quantities of BoAHV-1 virus. A synthetic DNA or DNA of known quantification cycle (Cq) value, extracted from a BoAHV-1 positive sample can be used as positive PCR control to check for PCR efficiency at each run and over time. A BoAHV-1 negative DNA or e.g. carrier DNA is used in the PCR as negative PCR control (no template control).

ii) Test results

Positive result: Any sample that has a cycle threshold (Ct) Cq value equal or less than 45 (Method 1) resp. 42 (Method 2) associated with an amplification curve is regarded as positive. The positive controls should have a Ct-Cq value within an acceptable range (± 3 Ct Cq values) as previously determined by repeatability testing. To minimise the risk of contamination by the positive controls, a dilution resulting in a Ct-Cq value of about 30 to 33 should be used.

Negative result: Any sample that shows no Ct-Cq value is regarded as negative. Negative control and no template controls should have no Ct-Cq values. Quality control of the extracted DNA and exclusion of PCR-inhibitors by internal or external control reactions are a prerequisite for the validation of a negative result.

1.4. Viral antigen detection

Nasal, ocular or genital swabs can be directly smeared onto glass cover-slips, or, following centrifugation, the cell deposit (see Section B.1.1) may be spotted onto cover-slips. These cover-slips are subjected to a standard direct or indirect fluorescent antibody test. In a direct immunofluorescence test, the monospecific antiserum is conjugated to a fluorescent dye e.g. fluorescein isothiocyanate (FITC) Alexa Fluor 488, whereas in the indirect procedure, the anti-species immunoglobulin secondary antibody is conjugated to a fluorochrome. To obtain reliable results, it is necessary to sample several animals in a herd that have fever and a slight, serous nasal discharge. Smears should be air-dried and fixed in acetone. Smears from nasal swabs from cattle with a purulent or haemorrhagic nasal discharge are often negative (~~Terpstra, 1979~~). The advantage of this antigen-detection technique is that it can lead to a same-day diagnosis. However, the sensitivity of this procedure is lower than that of virus isolation (~~Edwards et al., 1983~~) or PCR. Positive and negative controls must be included in each test.

Tissues collected at post-mortem can be examined for the presence of BoAHV-1 antigen by immunofluorescence analyses of frozen sections. Immunohistochemistry may also be applied for BoAHV-1 detection and determination of the antigen location in the tissues. MAbs are increasingly being used for detecting BoAHV-1 antigen, leading to enhanced specificity of the test. However, such MAbs must be carefully selected, because they must be directed against conserved epitopes that are present on all isolates of BoAHV-1.

Another possibility for direct rapid detection of viral antigen is the use of an enzyme-linked immunosorbent assay (ELISA). Antigen can be captured by MAbs or polyclonal antibodies coated on a solid phase, usually on microplates. Amounts of antigen equivalent to 10^4 – 10^5 TCID₅₀ of BoAHV-1 are required in order to obtain reliable positive results. This may not be unrealistically high, because titres of 10^8 – 10^9 TCID₅₀/ml of nasal fluid can be excreted by cattle 3–5 days after infection with BoAHV-1. Sensitivity can be increased by amplification systems (~~see Edwards & Citao, 1987~~).

In contrast to virus isolation, no cell culture facilities are required for direct antigen detection techniques and a laboratory diagnosis can be made within 1 day. The disadvantages are the lower sensitivity of direct antigen detection and the extra requirement to perform additional virus isolation, if the isolate is required for further studies. Therefore, real-time PCR is the state-of-the-art standard, and antigen detection should only be used if other methods are not available.

1.5. Differentiation of bovine herpesvirus 1 subtypes and of ruminant alphaherpesviruses related to bovine herpesvirus 1

By using appropriate MAbs for immunofluorescence, ~~radioimmuno-precipitation~~, immunoperoxidase or immunoblot assays, BoAHV-1 subtype 1 and subtype 2b can be differentiated (Rijsewijk *et al.*, 1999; ~~Wyller et al., 1989~~). Restriction endonuclease digestion of viral DNA enables differentiation between BoAHV-1 subtypes. RFLP analysis includes extraction of the DNA from virions or from infected cells, digestion of the isolated DNA by restriction endonucleases, and separation of the resulting fragments by agarose gel electrophoresis. Differentiation of the BoAHV-1 subtypes 1, 2a and 2b by HindIII

endonuclease digestion is based on the molecular weight of three relevant DNA fragments (*I*, *K* and *L*) (Metzler *et al.*, 1985). RFLP techniques are of limited diagnostic value, but may be useful in epidemiological studies. Furthermore, RFLP pattern of virus isolates can be compared with that of ~~live vaccine strains~~ the live vaccine strains. A multiplex PCR followed by restriction endonuclease analysis has also been applied for differentiation of BoAHV-1 subtypes (Maidana *et al.*, 2020). Phylogenetic comparison of the carboxy-terminal region of glycoprotein C (gC) is an additional promising tool for subtype differentiation of BoAHV-1.1, -1.2 and -5.

When differentiation is required between antigenically and genetically related ruminant alphaherpesviruses (BoAHV-1, BoAHV-5, caprine alphaherpesvirus 1, cervid alphaherpesvirus 1 and 2, elk alphaherpesvirus 1, bubaline alphaherpesvirus 1), improved methods are available using monoclonal antibodies (Keuser *et al.*, 2004) or PCR amplification and sequencing (~~Res *et al.*, 1999~~; Oberto *et al.*, 2023; Wernike *et al.*, 2011).

HTS techniques and related software can also be used to ~~reveal~~ analyse the whole genome of BoAHV-1 and compare different outbreak strains. The HTS is likely to be used more routinely due to improved technology ~~and~~ protocols and data analysis software. For optimised results, the virus titre should be as high as possible, and purified virus preparations allow the best sequencing results. See also Chapter 1.1.7 *Standards for high throughput sequencing, bioinformatics and computational genomics*.

1.6. Interpretation of results

The isolation of BoAHV-1 from a diseased animal does not unequivocally mean that this virus is the cause of the illness. It may, for instance, be a latent virus that has been reactivated due to stressful conditions. A confirmatory laboratory diagnosis must be made from a group of animals and must be accompanied by seroconversion from negative to positive, or a four-fold or higher increase in BoAHV-1-specific antibody titres. Paired serum samples collected 3–4 weeks apart are examined in a serological test for the presence of specific antibodies (see Section B.2).

2. Serological tests

Serological tests can be used for several purposes:

- i) To diagnose an acute infection: paired serum samples from the acute and convalescent stages of infection of the same animals are examined in one test. A seroconversion from negative to positive or a four-fold or higher increase in antibody titres is considered to prove an acute infection.
- ii) To demonstrate absence of infection, for instance, for international trade purposes.
- iii) To determine the prevalence of infection in sero-epidemiological studies.
- iv) To support eradication programmes and subsequent surveillance.
- v) For research purposes, for instance, the evaluation of the antibody response after vaccination and challenge infection.
- vi) To exclude BoAHV-1 infection in calves with maternal antibodies: paired serum samples drawn at least 4 weeks apart of the same animals are examined in one test. A four-fold or higher decrease in antibody titres is considered to pointing at a passively acquired immunity.

Virus neutralisation (~~VN~~) tests (~~Bitsch, 1978~~ VNT) and various ELISAs (~~Kramps *et al.*, 1993~~) are usually used for detecting antibodies against BoAHV-1 in serum. Because virus latency is a normal sequel to BoAHV-1 infection, the identification of serologically positive animals provides a useful and reliable indicator of infection status. Any animal with antibodies to the virus is considered to be a carrier and potential intermittent ~~excretor-shedder~~ of the virus. The only exceptions are calves that have acquired passive colostral antibodies from their dam, and noninfected cattle vaccinated with ~~inactivated~~ conventional modified live vaccines. It has been reported that, under experimental conditions, calves infected under cover of maternal immunity may become serologically negative while carrying a reactivatable latent infection (Lemaire *et al.*, 2000).

In general, BoAHV-1 serological tests can be divided into conventional and marker tests. Up to now, the only serological marker tests available are the BoAHV-1 gE-antibody blocking ELISAs (~~Van Oirschot *et al.*, 1997~~). Animals vaccinated with gE-deleted marker vaccines can be discriminated from field-virus infected animals by a negative serological reaction for gE. For conventional serology, VNT, BoAHV-1-antibody blocking ELISAs or indirect ELISAs may be used.

For the detection of antibodies in (bulk) milk samples ELISAs, including the gE-ELISA, are mainly used (~~Wellenberg *et al.*, 1998a~~), but have some limitations. By testing bulk milk, a positive gB-specific test indicates the presence of several infected animals in the herd (Frankena *et al.*, 1997). With the gE-blocking ELISA, bulk milk gives a positive

reaction only when more than 10–15% of the herd is infected (Wellenberg *et al.*, 1998b) although the sensitivity can be increased by milk concentration protocols (Schroeder *et al.*, 2012). Consequently, it is not possible to declare a herd to be free from BoAHV-1 infection with these tests on the basis of bulk or pooled milk samples, and a negative gE- or gB-ELISA bulk milk test should be followed up with individual blood samples from all cattle in the herd. However, indirect ELISAs optimised for use with bulk milk samples of up to 50 individual cows (or up to 100 animals in BoAHV-1-free regions) can indicate reliably the BoAHV-1 status of these animals. These test systems are able to detect one weak positive sample in a pool of 50 milk samples or one strong positive milk in a pool composed of 100 samples. For general surveillance purposes, bulk milk tank tests can give an estimate of BoAHV-1 prevalence in a herd, an area or country (Nylin *et al.*, 2000). These should be supplemented by serum testing (individual or pooled) from non-milking herds. For monitoring a BoAHV-1 status in dairy herds, bulk milk samples of up to 50 (or up to 100 in BoAHV-1-free regions) animals should be tested 3–4 times per year with a suitable indirect ELISA. In herds of more than 50 (or 100 in BoAHV-1-free regions) cows, several bulks of milk from the animals should be tested. Positive bulk milk results have to be confirmed by testing individual blood or milk samples from all animals that contributed to the positive bulk milk sample.

A very important occupational health and safety aspect is the possibility of testing adult fattening bulls by meat juice samples taken from the carcass after slaughter. Antibody contents are 10–30% reduced compared with blood samples. Therefore, a BoAHV-1 status definition by this method is restricted, but an estimation of IBR prevalence in beef cattle herds as well as survey of negative herds is enabled. Suitability of commercial ELISAs is extremely diverse and must be thoroughly evaluated. Further details should be tailored to the specific applications and can be requested from the WOA laboratory in Germany. In an extensive study, tests for the detection of antibodies routinely used by national reference laboratories in Europe were evaluated (Kramps *et al.*, 2004). Twelve reference laboratories from 12 European countries participated in this study. Fifty-three serum samples and 13 milk samples, originating from several countries, were sent in duplicate under code to the participating laboratories. The serum samples included the three European reference sera EU1 (antibody positive), EU2 (antibody weak positive and defined as borderline sample) and EU3 (antibody negative) (Perrin *et al.*, 1994). At that time, it was concluded that VNT and gB-specific ELISAs are the most sensitive tests for the detection of antibodies in serum. However, modern ELISA systems show a clearly improved sensitivity. Owing to the very high sensitivity of the gB-blocking ELISAs, gB-antibody weak positive results ~~can~~ could often not be confirmed by alternative test systems (indirect ELISA, VNT). Indirect BoAHV-1 ELISAs have now been developed that are also highly sensitive and specific. The results of these ELISAs are comparable with those obtained using gB-blocking ELISAs (Beer *et al.*, 2003).

gE-ELISAs are less sensitive ~~and specific~~ than the conventional test systems. In addition, seroconversion against gE can be delayed, especially in vaccinated animals, and is often not detectable before day 21 to 35 post-infection. ~~A minimum of three~~ Different commercial gE-ELISA systems ~~is~~ are currently available, allowing the analysis of samples with different gE-ELISAs each with unique characteristics.

2.1. Virus neutralisation test

VN tests (VNT) are performed with various modifications. ~~Tests~~ Testing protocols vary with regard to the virus strain used ~~in the test protocol~~, the starting dilution of the serum, the virus/serum incubation period (1–24 hours), the type of cells used, the day of final reading and the reading of the end-point (50% versus 100%) (Perrin *et al.*, 1993). Among these variables, the virus/serum incubation period has the most profound effect on the sensitivity of the VNT. A 24-hour incubation period may score up to 16-fold higher antibody titres than a 1-hour incubation period (Bitsch, 1978), and is recommended where maximum sensitivity is required (e.g. for international trade purposes). Various bovine cells or cell lines are suitable for use in the VNT, including secondary bovine kidney or testis cells, cell strains of bovine lung or tracheal cells, or the established MDBK cell line.

2.1.1. Suitable protocol for a VNT

- i) Inactivate sera, including control standard sera, for 30 minutes in a water bath at 56°C.
- ii) Make doubling dilutions of test sera in cell culture medium. Start with undiluted serum and continue to 1/1024 horizontally in a 96-well flat-bottomed cell-culture grade microtitre plate, at least three wells per dilution and 50 µl volumes per well. Dilutions of a positive control serum, and of weak positive and negative internal control sera, are also included in the test. An extra well with undiluted test serum is used for toxicity control of sera.
- iii) Add 50 µl per well of BoAHV-1 stock at a dilution in culture medium calculated to provide 100–200 TCID₅₀ per well. In the toxicity control wells, add 50 µl of culture medium in place of virus. Add 100 µl of culture medium to ten empty wells for cell controls.
- iv) Make at least four tenfold dilutions of the residual virus stock (back titration) in culture medium, using 50 µl per well and at least four wells per dilution.
- v) Incubate the plates for 24 hours at 37°C.

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- vi) Add 100 µl per well of the cell suspension (e.g. at 3×10^4 MDBK cells per well).
 - vii) Incubate the plates for 3–5 days at 37°C.
 - viii) Read the plates microscopically for CPEs. Validate the test by checking the back titration of virus (which should give a value of 100 TCID₅₀ with a permissible range of 30–300 TCID₅₀), the control sera and the cell control wells. The positive control serum should give a titre of ± 1 twofold dilution ($\pm 0.3 \log_{10}$ units) from its target value. The weak positive serum should be positive. The negative serum should give no neutralisation (equivalent to a final dilution of 1/2 at the neutralisation stage). In the cell control wells, the monolayers should be intact.
 - ix) The test serum results are expressed as the reciprocal of the dilution of serum that neutralised the virus in 50% of the wells. If 50% of the wells with undiluted serum neutralised the virus, the (initial dilution) titre is read as 1 (1/2 using the final dilution convention). If all the undiluted and 50% of the wells with 1/2 diluted serum neutralised the virus, the (initial dilution) titre is 2 (final dilution 1/4). For qualitative results, any neutralisation at a titre of 1 or above (initial dilution convention) is considered to be positive. If cytotoxicity is observed in the control wells, the sample is reported to be toxic (no result) unless neutralisation of the virus without cytotoxicity is observed at higher dilutions and a titre can be read without ambiguity. Where cytotoxicity of a serum interferes with the interpretation of the neutralising activity of the sample, changing the medium in the wells of the lowest two or three dilutions 16–24 hours after the addition of cells may remove the cytotoxic effects.

2.2. Enzyme-linked immunosorbent assay

ELISAs for the detection of ~~antibody~~ antibodies against BoA~~HV~~-1 are widely used, however a standard procedure for ELISA has not been established. Several types of ELISA are commercially available, including indirect and blocking ELISAs, some of which are also suitable for detecting antibodies in milk (Kramps *et al.*, 2004). For reasons of standardisation in a country or state, it may be desirable to compare the quality of the kits and to perform batch release tests by previously defined criteria in a national reference laboratory, before it is used by other laboratories in the country.

There are a number of variations in the ELISA procedures. The most common are: antigen preparation and coating, the dilution of the test sample, the incubation temperature and period of antigen and test sample, and the substrate/chromogen solution. Before being used routinely, an ELISA should be validated with respect to sensitivity, specificity and reproducibility (see chapter 1.1.6). For this purpose, a comprehensive panel of well defined (e.g. by VN test) strong positive, weak positive and negative sera has to be tested. However, it is recommended to use commercially available ELISAs that have been shown to perform better than home-made assays (Kramps *et al.*, 2004).

2.2.1. Indirect enzyme-linked immunosorbent assay

The principle of an indirect ELISA is based on the binding of BoA~~HV~~-1-specific antibodies present in the test sample to immobilised BoA~~HV~~-1 antigen. The bound antibodies are detected using enzyme-labelled anti-bovine immunoglobulin antiserum. The presence of antibodies in the test sample will result in colour development after addition of the substrate/chromogen solution. This test principle is the most suitable for individual and pooled milk samples.

2.2.2. Blocking enzyme-linked immunosorbent assay

The principle of a blocking or competitive ELISA is based on blocking the binding of an enzyme-labelled BoA~~HV~~-1 antiserum or anti-BoA~~HV~~-1 MAb to immobilised antigen by antibodies in the test sample. The presence of antibodies in the test sample results in reduced colour development after addition of the substrate/chromogen solution. An example of a gB blocking ELISA procedure is given below:

- i) Prepare the antigen by ~~growing-propagating~~ BoA~~HV~~-1 in cell cultures. When extensive CPE is observed, cells and medium are frozen at –20°C. After thawing, the resulting cellular lysate is centrifuged for 4 hours at 8500 *g*. The virus-containing pellet is suspended in a small volume of phosphate buffered saline (PBS), cooled on ice and disrupted using an ultrasonic disintegrator. The antigen preparation is then centrifuged for 10 minutes at 800 *g*, and inactivated by adding detergent (final concentration of 0.5% Nonidet P40). The antigen preparation is used at an appropriate dilution to coat the microtitre plates. Many alternative methods of antigen production are described in the published literature.
- ii) Coat the microtitre plates with antigen by adding 100 µl of diluted antigen (in 0.05 M carbonate buffer, pH 9.6) to each well. Seal the plates with tape, incubate at 37°C overnight, dry and store at –20°C.

- iii) Before the test is performed, wash the plates with 0.05% Tween 80. Add 100 µl negative serum (FCS), 100 µl of each of the serum test samples and 100 µl of positive, weak positive and negative control sera. Usually, serum samples are tested undiluted. Shake, seal the plates and incubate overnight at 37°C. If false-positive reactions are suspected (e.g. when epidemiologically implausible results are generated), it is recommended to retest such sera after heating for 30 minutes at 56°C following one freeze–thaw cycle.
- iv) Wash the plates thoroughly and add 100 µl of an anti-BoA_{HV}-1-gB-monoclonal antibody/horseradish peroxidase conjugate at a predetermined dilution, and incubate again for 1 hour at 37°C. The monoclonal antibody must be selected carefully for its specificity to gB of BoA_{HV}-1.
- v) Wash the plates and add freshly prepared substrate/chromogen solution (e.g. 0.05 M citric acid buffer, pH 4.5, containing 2,2'-azino-bis-[3-ethylbenzothiazoline]-6-sulphonic acid [ABTS; 0.55 mg/ml] and a 3% solution of freshly added H₂O₂ [5 µl/ml]), and incubate for the appropriate time (1–2 hours at room temperature).
- vi) Measure the absorbance of the plates on a microplate photometer at 405 nm.
- vii) Calculate for each test sample the blocking percentage $[(OD_{FCS} - OD_{test\ sample})/OD_{FCS} \times 100\%]$
- vii) A test sample is considered to be positive if it has a blocking percentage of e.g. 50% or higher. The test is valid if the positive and weak positive control sera are positive, and the negative control serum reacts negatively. The acceptable limits for control and cut-off values must be determined for the individual assay.

2.3. Standardisation

In each serological test, appropriate controls of strong positive, weak positive and negative serum should be included. A scientific group in Europe, initiated by the group of artificial insemination veterinarians of the European Union (EU), has agreed on the use of a strong positive (EU1), a weak positive (EU2) and negative serum (EU3) for standardisation of BoA_{HV}-1 tests in laboratories that routinely examine samples from artificial insemination centres (Perrin *et al.*, 1994). These sera have been adopted as WOA_H international standards for BoA_{HV}-1 tests and are available in limited quantities at the WOA_H Reference Laboratories for IBR/IPV⁵. Tests that are suitable for certifying individual animals prior to movement (VNT or ELISA) must be capable of scoring both the strong and weak positive standards (or secondary national standards of equivalent potency) as positive. Because of the limited availability of the international standard sera, there is a need to prepare a new extended panel of reference lyophilised serum (and milk) samples taken from infected as well as from vaccinated animals. This panel should be used to validate newly developed tests and to harmonise tests between laboratories. Additional reference sera are available in limited quantities from the WOA_H Reference Laboratories (e.g. R1, R2 and R3 as positive, weak positive and very weak positive standard sera from the WOA_H Reference Laboratory in Germany).

2.4. Nonspecific reactivity in BoA_{HV}-1-serology and 'pseudo-vaccines'

Nonspecific reactivity of sera in the BoA_{HV}-1-ELISAs should be taken into consideration₇ and is more often seen for the marker test than for the conventional serology. There are several reasons for nonspecific reactions:

- i) Batch variation of the ELISA used₂
- ii) Samples were tested very early after collection (freshness phenomenon)₂
- iii) Samples tested by gE-ELISA were collected within 4 weeks after vaccination with a marker vaccine (vaccination phenomenon)₂
- iv) Bad specimen quality (e.g. haemolysed samples).

Therefore₂ the following measures should be considered:

- i) Validation of each test batch, and batch release tests have to be implemented₂
- ii) Specimens should be stored at 4°C and should not be tested before 24–48 hours after sample collection₂

5 FLI, Greifswald-Insel Riems, Germany and APHA, Weybridge, UK. <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>

- iii) Samples should be subjected to a freeze–thaw cycle (–20°C); in some cases a subsequent heat inactivation (30 minutes/56°C) may eliminate nonspecific reactions of serum samples;
- iv) Cattle should not be serologically tested for BoAHV-1 prior to 4 weeks after any vaccination;
- v) gE-ELISAs should not be used for classification of unvaccinated animals.

In IBR/IPV-free regions, implausible singleton reactors are occasionally detected in gB blocking and indirect ELISAs, which cause problems concerning the status of both the animal and the affected herd. It has been suggested that BoAHV-2 infection could be the cause of this cross reactivity (Böttcher *et al.*, 2012). Interestingly, the gE-ELISAs are the most BoAHV-1-specific serological tests available and cross reactivity with non-BoAHV-1 herpesviruses is very low in these ELISAs. This is especially critical in BoAHV-1-free regions where single animals with cross reactivity are detected and might be misinterpreted as BoAHV-1 incursion into the herd. Therefore, in free regions, gE-ELISAs and VNT should be used to further verify such singleton reactors. One recommendation is to repeat testing of those animals after 28 days in a gE-ELISA using a lower cut-off (e.g. positive/negative of 0.95) to increase the sensitivity. In the case of two negative gE-ELISA results, the animal is not classified as BoAHV-1-positive, but it is recommended to slaughter the animal as diagnostic problems with other test systems may still occur (e.g. positive bulk milk results of the affected herd).

3. Emerging rapid diagnostics

Several IBR antibody rapid tests are commercially available. They can support monitoring of herd immunity, post-vaccination surveillance, epidemiological investigations, and contribute to disease control. However, the sensitivity and specificity of these pen-side tests are lower than those of approved ELISAs. Consequently, the reliable determination of the IBR infection status of individual animals must be verified by ELISAs.

Lateral flow devices (LFD) for pathogen detection are currently being developed as promising tools for the early IBR diagnosis on cattle farms. In particular, LFD-recombinase polymerase amplification assays (RPA) are rapid, robust, cost-effective, and sensitive. These assays are designed to detect BoAHV-1-specific nucleic acids (gC, gE, TK) following quick and simple DNA extraction and isothermal amplification. Portable and user-friendly systems have also been developed that are suitable for on-site diagnostic testing of clinical samples under resource-limited conditions. Advanced systems perform comparably to real-time PCR methods, though with slightly lower sensitivity, a critical factor in on-site quarantine testing and semen analysis. Consequently, positive test results generally have greater diagnostic value than negative results.

C. REQUIREMENTS FOR VACCINES

1. Background

1.1. Rationale and intended use of the product

Several attenuated and inactivated BoAHV-1 vaccines are currently available. The vaccine strains have usually undergone multiple passages in cell culture. Some of the vaccine virus strains have a temperature-sensitive phenotype, i.e. they do not replicate at temperatures of 39°C or higher. Attenuated vaccines are administered intranasally or intramuscularly. Inactivated vaccines contain high levels of inactivated virus or portions of the virus particle (glycoproteins) supplemented with an adjuvant to stimulate an adequate immune response. Inactivated vaccines are given intramuscularly or subcutaneously. Vaccination against BoAHV-1 is used to protect animals from the clinical outcome of infection, and as an aid-effective support in the control and eradication programmes.

Marker or DIVA (differentiation of infected from vaccinated animals) vaccines are now available in various countries. These attenuated or inactivated marker vaccines are based on deletion mutants (deletion of gE) or on a subunit of the virion, for instance glycoprotein D. The use of such marker vaccines in conjunction with companion diagnostic tests allows the distinction between infected and vaccinated cattle (DIVA principle), and provides the basis for BoAHV-1 eradication programmes in countries or regions with a high prevalence of field-virus infected animals. Intensive vaccination programmes can reduce the prevalence of infected animals (Bosch *et al.*, 1998; Mars *et al.*, 2001), which could be monitored by using an appropriate diagnostic test. In situations where it is economically justifiable, the residual infected animals could be slaughtered, resulting in a region free from BoAHV-1. Control and eradication of BoAHV-1 was started in some countries in the early 1980s. Different policies have been used due to differences in herd prevalence, breeding practices and disease eradication strategies. To date, in the EU, only gE-deleted DIVA vaccines (live as well as killed) have been marketed and used for control or eradication programmes.

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 of general nature and may be supplemented by national and regional requirements.

2. Outline of production and minimum requirements for conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

The vaccine is prepared using a seed-lot-like system. Origin, passage history and storage conditions of the master seed virus (MSV) must be recorded. A virus identity test must be performed on the MSV. The seed lot contains BoA₁HV-1 strains have to be attenuated to yield a live vaccine strain. The strains can be attenuated by multiple passages in cell cultures, by adapting virus to grow at low temperatures (temperature-sensitive mutants), or by genetic engineering, for example, by deleting one or more viral genes (e.g. the BoA₁HV-1 glycoprotein E) that are nonessential for replication. There should be some means of distinguishing the live vaccine virus from field viruses (for example temperature-specific growth patterns or restriction fragment length polymorphisms). Strains used for the preparation of inactivated vaccines need not be attenuated. The seed lot must be free from contaminants.

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

The seed lot is tested for absence of extraneous viruses and absence from contamination with bacteria, fungi or mycoplasma. The following extraneous viruses should be specifically excluded in BoA₁HV-1 vaccines: adenovirus, Akabane virus, Schmallenberg virus, bovine coronavirus, bovine herpesviruses 2, 4 and 5, bovine parvovirus, bovine respiratory syncytial virus, bovine viral diarrhoea virus and atypical pestiviruses, bovine rotavirus, vaccinia virus, and the viruses of Aujeszky's disease, bluetongue, bovine ephemeral fever, bovine leukaemia, bovine papilloma, bovine papular stomatitis, cowpox, foot and mouth disease, lumpy skin disease, malignant catarrhal fever, parainfluenza 3, rabies, rinderpest, and vesicular stomatitis. As bovine viral diarrhoea virus (either CPE or non-CPE) has regularly been found to be a contaminant of vaccines, special attention should be paid to the absence of BVDV. In addition, new atypical pestiviruses (e.g. HoBi or HoBi-like) have to be taken into consideration as possible contaminants.

2.2. Method of manufacture

2.2.1. Procedure

The cells used for vaccine production are prepared using a master-cell-lot system. The virus should be cultured on established cell lines that have been shown to be suitable for vaccine production, for example the Madin–Darby bovine kidney (MDBK) cell line. The history of the cell line must be known. The cell line must be free from extraneous agents and may be tested for tumorigenicity.

2.2.2. Requirements for substrates and media

All substances used for the manufacture of vaccines must be free from contaminants. Cells should be used that are not further than 20 passages from the master cell stock. The seed virus should not be more than five passages from the MSV. Genetically engineered vaccine virus strains are treated in the same way as conventionally attenuated vaccine virus strains. When sufficient cells are grown, infection of the cell line with the vaccine virus takes place. The addition of antibiotics is normally restricted to cell culture fluids. The supernatant fluid is harvested at times when the virus (antigen) production peaks. For live vaccines, the supernatant is clarified, mixed with a stabiliser, freeze-dried and bottled. For the production of classical inactivated vaccines, the supernatant is homogenised before the inactivating agent is added in order to ensure proper inactivation. The container should be changed to exclude survival of infectious agents on the lid and upper walls. After the inactivation procedure, a test for ensuring complete inactivation of the virus is carried out. The test should include at least two passages in cells. The inactivated virus suspension is then mixed with an adjuvant and bottled. The manufacture of vaccines must comply with guidelines for Good Manufacturing Practice (GMP) and local regulations such as European Pharmacopoeia or United States Code of Federal Regulations (9CFR).

2.2.3. In-process controls

Working cell seed and working virus seed must have been shown to be free from contaminants. The cells must show inconspicuous morphology before being inoculated with virus. The CPE is checked during cultivation. Uninoculated control cells must have retained their morphology until

the time of harvesting. A virus titration is performed on the harvested supernatant. During the production of inactivated vaccines, tests are performed to ensure inactivation. The final bulk must be tested for freedom from contaminants.

2.2.4. Final product batch tests

The following tests must normally be performed on each batch. Example guidelines for performing batch control can be found in EU directives, the European Pharmacopoeia and 9CFR.

i) Sterility/purity

Bacteria, fungi, mycoplasma and extraneous viruses must not be present. Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

ii) Safety

For inactivated vaccines, a twofold dose of vaccine, and for live vaccines, a tenfold dose of vaccine, must not produce adverse effects in young BoA_{HV}-1 seronegative calves.

iii) Batch potency

It is sufficient to test one representative batch for efficacy, as described in Section C.2.3.2. In the case of live vaccines, the virus titre of each batch must be determined and must be not higher than 1/10 of the dose at which the vaccine has been shown to be safe, and no lower than the minimum release titre. In the case of inactivated vaccines, the potency is tested using another validated method, for instance, efficacy assessment in calves.

2.3. Requirements for authorisation

2.3.1. Safety requirements

i) Target and non-target animal safety

A quantity of virus equivalent to ten doses of vaccine should (a) not induce significant local or systemic reactions in young calves; (b) not cause fetal infection or abortion, and (c) not revert to virulence during five serial passages in calves. For inactivated vaccine, a double dose is usually administered. The reversion to virulence test is not applicable to inactivated vaccines.

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

The selected final vaccine strain should not revert to virulence during a minimum of five serial passages in calves.

iii) Environmental consideration

Attenuated vaccine strains should not be able to perpetuate autonomously in a cattle population ($R_0 < 1$).

2.3.2. Efficacy requirements

i) For animal production

This must be shown in vaccination challenge experiments under laboratory conditions. Example guidelines are given in a monograph of the European Pharmacopoeia (Third Edition (1997–2025)). Briefly, the vaccine is administered to ten 2–3-month-old BoA_{HV}-1 seronegative calves. Two calves are kept as controls. All the calves are challenged intranasally 3 weeks later with a virulent strain of BoA_{HV}-1 that gives rise to typical clinical signs of a BoA_{HV}-1 infection. The vaccinated calves should show no or only very mild signs. The maximum (peak) virus titre in the nasal mucus of vaccinated calves should be at least 100 times lower than that in control calves. The virus excretion period should be at least 3 days shorter in vaccinated than in control calves.

An efficacious BoA_{HV}-1 vaccine should induce protective immunity for at least 1 year, although many existing vaccines have not been tested to this standard.

ii) For control and eradication

In addition to the above-mentioned criteria, Bo~~A~~HV-1-vaccines for control and eradication should be marker vaccines (e.g. gE-deleted vaccines) allowing the differentiation of infected from vaccinated animals (DIVA-strategy). Several gE-deleted vaccines (inactivated preparations as well as modified live vaccines) are commercially available.

2.3.3. Stability

For live vaccines, virus titrations should be carried out 3 months beyond the indicated shelf life. In addition, tests for determining moisture content, concentrations of preservatives, and pH are performed. For inactivated vaccines, the viscosity and stability of the emulsion are also tested.

The efficacy of preservatives should be demonstrated. The concentration of the preservative and its persistence throughout shelf life should be checked. The concentration must be in conformity with the limits set for the preservative.

3. Vaccines based on biotechnology

3.1. Vaccines available and their advantages

Glycoprotein E (gE)-deleted inactivated and modified live vaccines are available that are based on recombinant strains. The vaccines are comparable to other gE-deleted vaccines and are licensed by European Medicines Agency (EMA) for use in the EU.

Additional recombinant vaccines like gD-subunits or genetically engineered deletion mutants of Bo~~A~~HV-1 (e.g. with deletions of gE and/or gG) are described and available as prototypes.

Advantages of Bo~~A~~HV-1 vaccines based on biotechnology could be the possibility of additional marker features for the differentiation of infected from vaccinated animals (DIVA; e.g. gB-antibody-ELISAs for gD-subunit vaccines or gG-antibody-ELISAs for the respective deletion mutants).

3.2. Special requirements for biotechnological vaccines, if any

Recombinant vaccines, which are destined for use in the EU have to be licensed by EMA.

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NB: There are WOAH Reference Laboratories for infectious bovine rhinotracheitis/infectious pustular vulvovaginitis (please consult the WOAH Web site: <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3> <http://www.oie.int/>). Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for infectious bovine rhinotracheitis/infectious pustular vulvovaginitis

NB: FIRST ADOPTED IN 1990 AS INFECTIOUS BOVINE RHINOTRACHEITIS. MOST RECENT UPDATES ADOPTED IN 2017.

Appendix 1.
Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis
Intended purpose of test: population freedom from infection

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy (DSe/DSp)</u>	<u>Test population used to measure accuracy</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Indirect ELISA*</u> ☒ ☒ ☒ <u>Bovine</u>	<u>Blood and milk</u> <u>Antibodies to the whole virus or viral proteins (e.g. gC, gE)</u>	<u>Blood:</u> <u>DSe ~98%</u> <u>DSp ≥99.5%</u> <u>Milk:</u> <u>DSe ≥95%</u> <u>DSp ≥99%</u>	<u>Certified free or positive herds (VN as gold standard, defined populations in eradication programmes)</u>	<u>Manufacturers and licensing authorities (details on request)</u>	<u>- Detects latently infected animals</u> <u>- Commercially available</u> <u>- Updated generations with enhanced sensitivity and specificity</u> <u>- Universal detectability of all BoAHV-1 strains</u>	<u>- Compared with blocking ELISA slightly reduced specificity and sensitivity</u> <u>- In single cases may cross react with BoAHV-2</u> <u>- Antigen dependent general cross reactivity with related ruminant alphaherpesviruses</u>	<u>Perrin <i>et al.</i> (1993)</u> <u>Kramps <i>et al.</i> (2004)</u>
<u>Blocking ELISA*</u> ☒ ☒ ☒ <u>Susceptible species</u>	<u>Blood and milk</u> <u>Antibodies to viral proteins e.g. gB, gE</u>	<u>DSe ~99%</u> <u>DSp 99.7%</u>	<u>Certified free or positive herds (VN as gold standard, defined populations in eradication programmes)</u>	<u>Manufacturers and licensing authorities (details on request)</u>	<u>- Detects latently infected animals</u> <u>- Commercially available</u> <u>- Compared with indirect ELISA, enhanced specificity and slightly enhanced sensitivity</u> <u>- Conventional and marker diagnostics possible</u> <u>- Milk testing at enhanced frequencies / concentration systems available</u> <u>- Universal detectability of all BoAHV-1 strains (exception: marker ELISAs)</u> <u>- gE-ELISA: lower cross reactivity compared with gB, DIVA successful application in practice</u>	<u>- In single cases may cross react with BoAHV-2</u> <u>- Antigen dependent general cross reactivity with related ruminant alphaherpesviruses</u> <u>- Reduced sensitivity for milk testing (threshold >10–20% of positive animals in the pool)</u> <u>- gE-ELISA: reduced sensitivity compared with gB, late onset of immune response</u> <u>- Not suitable for bulk milk without concentration</u>	<u>Schroeder <i>et al.</i> (2012)</u> <u>Kalthoff <i>et al.</i> (2010)</u>
<u>VNT</u> ☒ ☒ ☐ <u>Susceptible species</u>	<u>Serum</u> <u>Antibodies to the whole virus</u>	<u>DSe ≤9%</u> <u>DSp towards 100%</u>	<u>Certified free or positive herds (ELISA classification, defined populations in eradication programmes)</u>	<u>Proficiency testing (details on request)</u>	<u>- Detection of latently infected animals</u> <u>- Nearly absolute specificity</u> <u>- Cross reactivity can be used to differentiate between related ruminant herpesviruses (≥log₂ titre difference)</u> <u>- Species independent</u> <u>- Universal detectability for all BoAHV-1 strains</u>	<u>- Time consuming, not recommended for mass testing</u> <u>- Cell culture dependent</u> <u>- Lower sensitivity compared with ELISA techniques</u>	

*A general statement on DSe/DSp of commercial ELISAs is not possible: performance could be varied with substantial differences in the test quality. DSe/DSp might be quite low in a variety of commercial or even in-house tests. Choice of the test is dependent on test purpose (e.g. screening or determination of an individual status).

Appendix 2.
Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis
Intended purpose of test: individual animal freedom from infection prior to movement

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy (DSe/DSp)</u>	<u>Test population used to measure accuracy</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Virus isolation</u> ☒ ☐ ☐ <u>Bovine</u>	<u>Semen (Individual animals)</u> <u>Infectious virus</u>	<u>DSe ~95%</u> <u>DSp up to 100%</u> <u>(confirmation with IFT, real-time PCR)</u>	<u>Certified free or positive herds (eradication programmes), animal experiments, collection of virus strains</u>	<u>See references</u>	- Generation of virus isolates for further characterisation: subtyping, molecular epidemiology (HTS, RFLP) - Conventional and marker diagnostics possible - Species independent	- Lower sensitivity especially at the end phase of acute infection (presence of neutralising antibodies) - Time consuming and labour intensive - Cell culture dependent (experience necessary) - Limited use and applicable only to diseased animal as infection is often without clinical signs - Not suitable for animals in a latent phase of infection	<u>Abril <i>et al.</i> (2004)</u> <u>Wang <i>et al.</i> (2008)</u> <u>Wernike <i>et al.</i> (2011)</u> <u>Van Engelenburg <i>et al.</i> (1995)</u>
<u>Real-time PCR</u> ☒ ☒ ☐ <u>Bovine</u>	<u>Semen (Individual animals)</u> <u>Viral genome sequences e.g. gB, gD and gE</u>	<u>DSe ~99%</u> <u>Specificity depends on selected target gene</u> <u>Cross reactivity with ruminant alphaherpesviruses possible</u>	<u>Certified free or positive herds (eradication programmes), animal experiments, collection of virus strains</u>	<u>See references</u>	- Commercially available - Targets highly conserved - Genetic DIVA possible - Species independent - Prolonged genome detection in the acute phase of infection compared with VI	- Prone to contamination - Cross reactivity with related ruminant alphaherpesviruses possible - Elaborated equipment necessary, consumables may be not readily available - Limited use and applicable only to diseased animal as infection is often without clinical signs - Not suitable for animals in a latent phase of infection	<u>Abril <i>et al.</i> (2004)</u> <u>Wang <i>et al.</i> (2008)</u> <u>Wernike <i>et al.</i> (2011)</u>
<u>Indirect ELISA*</u> ☒ ☒ ☒ <u>Bovine</u>	<u>Blood</u> <u>Antibodies to the whole virus or viral proteins (e.g. gC, gE)</u>	<u>DSe ~98%</u> <u>DSp ≥99.5%</u> <u>DSe ≥95%</u> <u>DSp ≥99%</u>	<u>Certified free or positive herds (VN as gold standard, defined populations in eradication programmes)</u>	<u>Manufacturers and licensing authorities (details on request)</u>	- Detects latently infected animals - Commercially available - Updated generations with enhanced sensitivity and specificity - Universal detectability of all BoAHV-1 strains	- Compared with blocking ELISA slightly reduced specificity and sensitivity - In single cases may cross react with BoAHV-2 - Antigen dependent general cross reactivity with related ruminant alphaherpesviruses - Applicable only to naïve, unvaccinated animals	<u>Perrin <i>et al.</i> (1993)</u> <u>Kramps <i>et al.</i>, (2004)</u>

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy (DSe/DSp)</u>	<u>Test population used to measure accuracy</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Blocking ELISA*</u> ☒ ☒ ☒ <u>Susceptible species</u>	<u>Blood and milk</u> <u>Competitive antibodies to viral proteins e.g. gB, gE</u>	<u>DSe ~99%</u> <u>DSp 99.7%</u>	<u>Certified free or positive herds (VN as gold standard, defined populations in eradication programmes)</u>	<u>Manufacturers and licensing authorities (details on request)</u>	- Detects latently infected animals - Commercially available - Compared with indirect ELISA enhanced specificity and slightly enhanced sensitivity - Conventional and marker diagnostics possible - Universal detectability of all BoAHV-1 strains (exception: marker ELISAs) - gE-ELISA: lower cross reactivity compared with gB, DIVA	- May in single cases cross react with BoAHV-2 - Antigen dependent general cross reactivity with related ruminant alphaherpesviruses - Reduced sensitivity for milk testing (threshold >10-20% of positive animals in the pool) - gE-ELISA: reduced sensitivity compared with gB, late onset of immune response, low percentage of vaccinated animals might show no gE-seroconversion after field virus infection - Applicable only to naïve animals and gE-marker vaccinated animals	<u>Schroeder <i>et al.</i> (2012)</u> <u>Kalthoff <i>et al.</i> (2010)**</u>
<u>VNT</u> ☒ ☒ ☐ <u>Bovine</u>	<u>Serum</u> <u>Antibody to the whole virus</u>	<u>DSe ≤90%</u> <u>DSp towards 100%</u>	<u>Certified free or positive herds (ELISA classification, defined populations in eradication programmes)</u>	<u>Proficiency testing (details on request)</u>	- Detection of latently infected animals - Nearly absolute specificity - Cross reactivity can be used to differentiate between related ruminant herpesviruses (≥log2 titer difference) - Species independent - Universal detectability for all BoAHV-1 strains	- Time consuming - Cell culture dependent - Lower sensitivity compared with ELISA techniques - Applicable only to naïve animals	

*A general statement on DSe/DSp of commercial ELISAs is not possible: performance could be varied with substantial differences in the test quality, DSe/DSp might be quite low in a variety of commercial or even in-house tests. Choice of the test is dependent on test purpose (e.g. screening or determination of an individual status).

Appendix 3.
Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis
Intended purpose of test: contribute to eradication policies; prevalence of infection - surveillance

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy (DSe/DSp)</u>	<u>Test population used to measure accuracy</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Virus isolation</u> ☒ ☐ ☐ <u>Susceptible species</u>	<u>Nasal and genital swabs, various organ samples at post-mortem</u> <u>Semen</u> <u>Infectious virus</u>	<u>DSe ~95%</u> <u>DSp up to 100%</u> <u>(confirmation with IFT, qPCR)</u>	<u>Certified free or positive herds (eradication programmes), animal experiments, collection of virus strains</u>	<u>See references</u>	<u>- Generation of virus isolates for further characterisation: subtyping, molecular epidemiology (HTS, RFLP)</u> <u>- Conventional and marker diagnostics possible</u> <u>- Species independent</u> <u>- Active and passive monitoring, early detection of the infection</u>	<u>- Lower sensitivity especially at the end phase of acute infection (presence of neutralising antibodies)</u> <u>- Time consuming and labour intensive</u> <u>- Cell culture dependent (experience necessary)</u>	<u>Abril <i>et al.</i> (2004)</u> <u>Wang <i>et al.</i> (2008)</u> <u>Vernike <i>et al.</i> (2011)</u> <u>Van Engelenburg <i>et al.</i> (1995)</u>
<u>Real-time PCR</u> ☒ ☐ ☐ <u>Susceptible species</u>	<u>Nasal and genital swabs, various organ samples at post-mortem</u> <u>Semen</u> <u>Viral genome sequences e.g. gB, gD and gE</u>	<u>DSe ~99%</u> <u>Specificity depends on selected target gene</u>	<u>Certified free or positive herds (eradication programmes), animal experiments, collection of virus strains</u>	<u>See references</u>	<u>- Commercially available</u> <u>- Targets highly conserved</u> <u>- Genetic DIVA possible</u> <u>- Species independent</u> <u>- Prolonged genome detection in the acute phase of infection compared with VI</u> <u>- Epidemiological investigation possible</u>	<u>- Prone to contamination</u> <u>- Cross reactivity with related ruminant alphaherpesviruses possible</u> <u>- Elaborated equipment necessary, consumables may be not readily available</u> <u>- Cross reactivity with ruminant alphaherpesviruses possible</u>	<u>Abril <i>et al.</i> (2004)</u> <u>Wang <i>et al.</i> (2008)</u> <u>Vernike <i>et al.</i> (2011)</u>
<u>ELISA*</u> ☒ ☒ ☒ <u>Bovine</u>	<u>Blood</u> <u>Antibody to the whole virus, gB and gE</u>	<u>DSe ≥95–99%</u> <u>DSp ≥99.0–99.7%</u>	<u>Certified free or positive herds (VN as gold standard, defined populations in eradication programmes)</u>	<u>Manufacturers and licensing authorities (details on request)</u>	<u>See Appendix 1</u>	<u>See Appendix 1</u> <u>Seroconversion not earlier than day 7</u> <u>Additional testing of a contact group is recommended</u>	<u>Perrin <i>et al.</i> (1993)</u> <u>Kramps <i>et al.</i> (2004)</u> <u>Kalthoff <i>et al.</i> (2010)</u>
<u>VNT</u> ☒ ☒ ☐ <u>Susceptible species</u>	<u>Serum</u> <u>Antibodies to the whole virus</u>	<u>DSe ≤90%</u> <u>DSp towards 100%</u>	<u>Certified free or positive herds (ELISA classification, defined populations in eradication programmes)</u>	<u>Proficiency testing (details on request)</u>	<u>See Appendix 1</u>	<u>- Time consuming, not recommended for mass testing</u> <u>- Cell culture dependent</u> <u>- Lower sensitivity compared with ELISA techniques</u>	

*A general statement on DSe/DSp of commercial ELISAs is not possible: performance could be varied with substantial differences in the test quality. DSe/DSp might be quite lower in a variety of commercial or even in-house tests. Choice of the test is dependent on test purpose (e.g. screening or determination of an individual status).

Appendix 4.
Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis
Intended purpose of test: confirmation of clinical cases

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy (DSe/DSp)</u>	<u>Test population used to measure accuracy</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Virus isolation</u> ☒ ☐ ☐ <u>Susceptible species</u>	<u>Nasal and genital swabs, various organs at post-mortem, aborted materials</u> <u>Infectious virus</u>	<u>DSe ~95%</u> <u>DSp up to 100%</u> <u>(confirmation with IFT, real-time PCR)</u>	<u>Certified free or positive herds (eradication programmes), animal experiments, collection of virus strains</u>	<u>See references</u>	<u>- Generation of virus isolates for further characterisation: subtyping, molecular epidemiology (HTS, RFLP)</u> <u>- Conventional and marker diagnostics possible</u> <u>- Species independent</u>	<u>- Lower sensitivity especially at the end phase of acute infection (presence of neutralising antibodies)</u> <u>- Time consuming and labor intensive</u> <u>- Cell culture dependent (experience necessary)</u>	<u>Abril <i>et al.</i> (2004)</u> <u>Wang <i>et al.</i> (2008)</u> <u>Wernike <i>et al.</i> (2011)</u> <u>Van Engelenburg <i>et al.</i> (1995)</u>
<u>Real-time PCR</u> ☒ ☒ ☒ <u>Susceptible species</u>	<u>Nasal and genital swabs, various organs at post-mortem, aborted materials</u> <u>Viral genome sequences e.g. gB, gD and gE</u>	<u>DSe ~99%</u> <u>Specificity depends on selected target gene</u> <u>Cross reactivity with ruminant alphaherpesviruses possible</u>	<u>Certified free or positive herds (eradication programmes), animal experiments, collection of virus strains</u>	<u>See references</u>	<u>- Detects latently infected animals</u> <u>- Commercially available</u> <u>- Targets highly conserved</u> <u>- Genetic DIVA possible</u> <u>- Species independent</u> <u>- Prolonged genome detection in the acute phase of infection compared with VI</u> <u>- Epidemiological investigation possible</u>	<u>- Prone to contamination</u> <u>- Cross reactivity with related ruminant alphaherpesviruses possible</u> <u>- Elaborated equipment necessary, consumables may be not readily available</u>	<u>Abril <i>et al.</i> (2004)</u> <u>Wang <i>et al.</i> (2008)</u> <u>Wernike <i>et al.</i> (2011)</u>
<u>ELISA*</u> ☒ ☒ ☐ <u>Indirect ELISA: Bovine</u> <u>-Blocking ELISA: Susceptible species</u>	<u>Blood</u> <u>Antibody to the whole virus, gB and gE</u>	<u>DSe ≥95–99%</u> <u>DSp ≥99.0–99.7%</u>	<u>Certified free or positive herds (VN as gold standard, defined populations in eradication programmes)</u>	<u>Manufacturers and licensing authorities (details on request)</u>	<u>See Appendix 1</u> <u>Additional testing for confirmatory purposes:</u> <u>- Blood samples from contact animals</u> <u>- Blood sample of the mother in case of abortion</u> <u>- Examination of paired serum samples indicative for recent infection.</u>	<u>See Appendix 1</u> <u>- Seroconversion not earlier than day 7</u>	<u>Perrin <i>et al.</i> (1993)</u> <u>Kramps <i>et al.</i>, (2004)</u> <u>Kalthoff <i>et al.</i> (2010)</u>
<u>VNT</u> ☒ ☐ ☐ <u>Susceptible species</u>	<u>Serum</u> <u>Antibody to the whole virus</u>	<u>DSe ≤90%</u> <u>DSp towards 100%</u>	<u>Certified free or positive herds (ELISA classification, defined populations in eradication programmes)</u>	<u>Proficiency testing (details on request)</u>	<u>See Appendix 1</u> <u>Additional testing for confirmatory purposes:</u> <u>- Blood samples from contact animals</u> <u>- Blood sample of the mother in case of abortion</u> <u>- Examination of paired serum samples indicative for recent infection</u>	<u>See Appendix 1</u> <u>- Seroconversion not earlier than day 7–10</u> <u>- Less sensitive than ELISA</u>	

*A general statement on DSe/DSp of commercial ELISAs is not possible: performance could be varied with substantial differences in the test quality. DSe/DSp might be quite lower in a variety of commercial or even in-house tests. Choice of the test is dependent on test purpose (e.g. screening or determination of an individual status).

Appendix 5.
Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis
Intended purpose of test: immune status in individual animals or populations post-vaccination

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy (DSe/DSp)</u>	<u>Test population used to measure accuracy</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>ELISA*</u> ☒ ☒ ☒ <u>-Indirect ELISA:</u> <u>Bovine</u> <u>-Blocking ELISA:</u> <u>Susceptible species</u>	<u>Blood</u> <u>Antibody to the whole virus, viral proteins e.g. gB and gC</u>	<u>DSe ≥95–99%</u> <u>DSp ≥99.0–99.7%</u>	<u>Certified free or positive herds (VN as gold standard, defined populations in eradication programmes)</u>	<u>Manufacturers and licensing authorities (details on request)</u>	<u>- Commercially available</u> <u>- Conventional and marker diagnostics possible</u> <u>- Detection of latently infected animals</u> <u>- DIVA diagnostics with gE marker ELISA</u> <u>- Immune status correlates to clinical protection</u>	<u>- Indirect ELISA: compared with blocking ELISA slightly reduced specificity and sensitivity</u> <u>- gE-ELISA: reduced sensitivity compared with gB, late onset of immune response, low percentage of vaccinated animals might show no gE-seroconversion after field virus infection</u> <u>- No differentiation between infected and conventionally vaccinated animals possible</u>	<u>Perrin <i>et al.</i> (1993)*</u> <u>Kramps <i>et al.</i> (2004)</u> <u>Kalthoff <i>et al.</i> (2010)**</u>
<u>VNT</u> ☒ ☒ ☐ <u>Susceptible species</u>	<u>Serum</u> <u>Antibody to the whole virus</u>	<u>DSe ≤90%</u> <u>DSp towards 100%</u>	<u>Certified free or positive herds (ELISA classification, defined populations in eradication programmes)</u>	<u>Proficiency testing (details on request)</u>	<u>See Appendix 1</u> <u>- Immune status correlates to clinical protection</u>	<u>- No reliable differentiation between infected and vaccinated animals possible</u>	

*A general statement on DSe/DSp of commercial ELISAs is not possible: performance could be varied with substantial differences in the test quality. DSe/DSp might be quite lower in a variety of commercial or even in-house tests. Choice of the test is dependent on test purpose (e.g. screening or determination of an individual status).

MEETING OF THE BIOLOGICAL STANDARDS COMMISSION

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CHAPTER 3.5.1.

CAMELPOX

SUMMARY

*Camelpox is a wide-spread infectious viral disease of ~~Old World~~ camelids in the genus Camelus. ~~New World camelids~~ Llamas, alpacas, guanacos and vicunas (genus Llama and Vicugna) are also susceptible. It occurs throughout the camel-breeding areas of Africa, north of the equator, the Middle East and Asia, and has an important economic impact through loss of production and sometimes death. Camelpox does not occur in the feral camel population of Australia. Camelpox virus belongs to the family Poxviridae, subfamily Chordopoxvirinae, genus Orthopoxvirus. The disease is characterised by fever, local or generalised pox lesions on the skin and in the mucous membranes of the mouth and respiratory tract. The clinical manifestations range from inapparent infection to mild, moderate and, less commonly, severe systemic infection and death. The disease occurs more frequently and more severely in young animals and pregnant females. Transmission is by either direct contact between infected and susceptible animals or indirect infection via a contaminated environment. The role of insects in transmission has been suspected because the disease is often observed after rainfall. Camelpox virus is very host specific and does not infect other animals. Zoonotic camelpox virus infection in humans associated with outbreaks in dromedary camels (*Camelus dromedarius*) was described in the north-eastern region of India during 2009. This was a single incident illustrating that camelpox is of limited public health importance.*

Detection of the agent: *The presumptive diagnosis of camelpox infection is based on clinical signs. However, infections of camels with contagious ecthyma (orf), papilloma virus and reaction to insect bites are considered differential diagnoses in the early clinical stages and in mild cases of camelpox. Several diagnostic methods are available and, where possible, more than one should be used to make a confirmatory diagnosis of disease.*

The preferred method of laboratory confirmation of camelpox is by the use of polymerase chain reaction (PCR) methods. Alternatively, the characteristic, brick-shaped orthopoxvirions can be demonstrated in skin lesions, scabs or tissue samples using transmission electron microscopy (TEM). Camelpox virus is distinct from the ovoid-shaped parapox virus, the aetiological agent of the principle differential diagnosis: camel orf. However, both viruses may be seen simultaneously by TEM as dual infections.

Camelpox can be confirmed by demonstration of the camelpox antigen in scabs and pock lesions in tissues by immunohistochemistry. It is a relatively simple method that can be performed in laboratories where TEM is not available. In addition, the paraffin-embedded samples can be stored for a long period of time, enabling future epidemiological, retrospective studies.

Camelpox virus may be propagated on the chorioallantoic membrane (CAM) of embryonated chicken eggs. After 5 days, characteristic lesions can be observed on the CAM. Camelpox virus shows typical cytopathic effect on a wide variety of cell cultures. Intracytoplasmic eosinophilic inclusion bodies, characteristic of poxvirus infection, may be demonstrated in infected cells using haematoxylin and eosin staining. The presence of viral nucleic acid may be confirmed by PCR, and different strains of camelpox virus may be identified using DNA restriction enzyme analysis. An antigen-capture enzyme-linked immunosorbent assay (ELISA) for the detection of camelpox virus has been described.

Serological tests: *A wide range of serological tests is available to identify camelpox, including virus neutralisation and ELISA.*

Requirements for vaccines: Both attenuated and inactivated vaccines are commercially available. Vaccination with live attenuated vaccine provides protection for at least 6 years and with inactivated vaccine for 12 months.

A. INTRODUCTION

Camelpox occurs in almost every country in which camel husbandry is practised apart from the introduced dromedary camel in Australia and tylopods (llama and related species) in South America. Outbreaks have been reported in the Middle East, Asia and Africa (Mayer & Czerny, 1990; Wernery *et al.*, 1997b). The disease is endemic in many countries, and a pattern of sporadic outbreaks occurs with a rise in the seasonal incidence usually during the rainy season. For up-to-date information, consult WOAHA WHIS interface¹).

Camelpox is caused by *Camelpox virus*, which belongs to the genus *Orthopoxvirus* within the family *Poxviridae*. Based on sequence analysis, it has been determined that the camelpox virus is the most closely related to variola virus, the aetiological agent for smallpox. Camels have been successfully vaccinated against camelpox with vaccinia virus strains. The average size of the virion is 265–295 nm. Orthopoxviruses are enveloped, brick-shaped and the outer membrane is covered with irregularly arranged tubular proteins. A virion consists of an envelope, outer membrane, two lateral bodies and a core. The double-stranded DNA genome is approximately 206 kbp. Virus replicates in the cytoplasm of the host cell, in so-called inclusion bodies. Camelpox virus haemagglutinates chicken erythrocytes, but the haemagglutination may be poor (Davies *et al.*, 1975). Camelpox virus is ether resistant and chloroform sensitive (Davies *et al.*, 1975; Tantawi *et al.*, 1974). The virus is sensitive to pH 3–5 and pH 8.5–10 (Davies *et al.*, 1975). Poxviruses are susceptible to various disinfectants including 1% sodium hypochlorite, 1% sodium hydroxide, 1% peracetic acid, 0.5–1% formalin and 0.5% quaternary ammonium compounds. The virus can be destroyed by autoclaving or boiling for 10 minutes and is killed by ultraviolet rays (245 nm wavelength) in a few minutes (Coetzer, 2004).

The incubation period is usually 9–13 days (varying between 3 and 15 days). Clinical manifestations of camelpox range from inapparent and mild local infections, confined to the skin, to moderate and severe systemic infections, possibly reflecting differences between the strains of camelpox or differences in the immune status of the animals (Wernery *et al.*, 2014). The disease is characterised by fever, enlarged lymph nodes and skin lesions. Skin lesions appear 1–3 days after the onset of fever, starting as erythematous macules, developing into papules and vesicles, and later turning into pustules. Crusts develop on the ruptured pustules. These lesions first appear on the head, eyelids, nostrils and the margins of the ears. In severe cases the whole head may be swollen. Later, skin lesions may extend to the neck, limbs, genitalia, mammary glands and perineum. In the generalised form, pox lesions may cover the entire body. Skin lesions may take up to 4–6 weeks to heal. In the systemic form of the disease, pox lesions can be found in the mucous membranes of the mouth and respiratory tract (Kritz, 1982; Wernery *et al.*, 2014).

The animals may show salivation, lacrimation and a mucopurulent nasal discharge. Diarrhoea and anorexia may occur in the systemic form of the disease. Pregnant females may abort. Death is usually caused by secondary infections and septicæmia (Wernery *et al.*, 2014).

Histopathological examination of the early skin nodules reveals characteristic cytoplasmic swelling, vacuolation and ballooning of the keratinocytes of the outer stratum spinosum. The rupture of these cells produces vesicles and localised oedema. Perivascular infiltration of mononuclear cells and variable infiltration of neutrophils and eosinophils occurs. Marked epithelial hyperplasia may occur in the borders of the skin lesions (Yager *et al.*, 1991).

There are only a few detailed pathological descriptions of internal camelpox lesions. The lesions observed on post-mortem examination of camels that die following severe infection with camelpox are multiple pox-like lesions on the mucous membranes of the mouth and respiratory tract. The size of the lesions in the lungs may vary in diameter between 0.5 and 1.3 cm, occasionally up to 4–5 cm. Smaller lesions may have a haemorrhagic centre. The lung lesions are characterised by hydropic degeneration, proliferation of bronchial epithelial cells, and infiltration of the affected areas by macrophages, necrosis and fibrosis (Kinne *et al.*, 1998; Pfeffer *et al.*, 1998a; Wernery *et al.*, 1997a). Pox lesions are also observed in the mucosa of the trachea and retina of the eye causing blindness.

The morbidity rate of camelpox is variable and depends on whether the virus is circulating in the herd. Serological surveys taken in several countries reveal a high prevalence of antibodies to camelpox (Wernery *et al.*, 2014). The incidence of disease is higher in males than females, and the mortality rate is greater in young animals than in adults (Kritz, 1982). The mortality rate in adult animals is between 5% and 28% and in young animals between 25% and 100% (Mayer & Czerny, 1990).

1 <http://www.woah.org/en/animal-health-in-the-world/the-world-animal-health-information-system/the-world-animal-health-information-system/>

Transmission is by either direct contact between infected and susceptible animals or indirect infection via a contaminated environment. The infection usually occurs by inhalation or through skin abrasions. Virus is secreted in milk, saliva, and ocular and nasal discharges. Dried scabs shed from the pox lesions may contain live virus for at least 4 months and contaminate the environment. The role of an arthropod vector in the transmission of the disease has been suspected. Camelpox virus has been detected by transmission electron microscopy (TEM) and virus isolation from the camel tick, *Hyalomma dromedarii*, collected from animals infected with camelpox virus. The increased density of the tick population during the rainy season may be responsible for the spread of the disease (Wernery *et al.*, 1997a). However, other potential vectors may be involved, such as biting flies and mosquitoes.

Different strains of camelpox virus may show some variation in their virulence (Wernery *et al.*, 2014). Restriction enzyme analysis of viral DNA allows isolates to be compared. However, no major differences from the vaccine strain have so far been demonstrated (Wernery *et al.*, 1997a).

Immunity against camelpox is both humoral and cell mediated. The relative importance of these two mechanisms is not fully understood, but it is believed that circulating antibodies do not reflect the immune status of the animal (Wernery *et al.*, 2014). Life-long immunity follows after natural infection. Live, attenuated vaccine provides protection against the disease for at least 6 years, probably longer (Wernery & Zachariah, 1999). Inactivated vaccine provides protection for 1 year only.

The camelpox virus is very host specific and does not infect other animal species, including cattle, sheep and goats. Several cases in humans have been described (Kritz, 1982); the most recent from India (Bera *et al.*, 2011) with clinical manifestations such as papules, vesicles, ulceration and finally scabs on fingers and hands. However, such cases and even milder human infections seem to be rare illustrating that camelpox is of limited public health importance. Camelpox virus should be handled with appropriate biosafety and biocontainment measures 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 camelpox and their purpose

Method	Purpose					
	Population freedom from infection ^(a)	Individual animal freedom from infection prior to movement ^(b)	Contribute to eradication policies	Confirmation of clinical cases ^(c)	Prevalence of infection – surveillance ^(d)	Immune status in individual animals or populations post-vaccination ^(e)
Detection and identification of the agent^(f)						
TEM	□□□	□□□	□□□	☑☑☑	□□□	□□□
Virus isolation in cell culture	□□□	□□□	□□□	☑☑☑	□□□	□□□
Virus isolation on CAM	□□□	□□□	□□□	☑☑☑	□□□	□□□
Immuno-histochemistry	□□□	□□□	□□□	☑☑☑	□□□	□□□
PCR	□□□	□□□	□□□	☑☑☑	□□□	□□□
Real-time PCR	□□□	□□□	□□□	☑☑☑	□□□	□□□
Detection of immune response						
ELISA	☑☑☑	☑☑☑-☑□□	□□□	☑□□	☑☑☑	☑☑☑
Virus neutralisation	☑☑☑	☑☑☑-☑□□	□□□	☑□□	☑☑☑	☑☑☑

Key: ☑☑☑ = recommended for this purpose; ☑☑□ = recommended but has limitations;

☑□□ = suitable in very limited circumstances; □□□ = not appropriate for this purpose.

TEM: = Transmission electron microscopy; CAM = chorioallantoic membrane of embryonated chicken eggs;

PCR = polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay.

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

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

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

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

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

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

During the viraemic stage of the disease (within the first week of the occurrence of clinical signs) camelpox virus can be isolated in cell culture from heparinised blood samples, or viral DNA can be detected by the polymerase chain reaction (PCR) from blood in EDTA (ethylene diamine tetra-acetic acid). The blood samples should be collected in a sterile manner by venepuncture. Blood samples, with anticoagulant for virus isolation from the buffy coat, should be placed immediately on ice and processed as soon as possible. In practice, the samples can be kept at 4°C for up to 2 days prior to processing, but should not be frozen or kept at ambient temperatures.

Blood obtained for serum samples should be collected in plain tubes with no anticoagulant. ~~The Blood tubes samples~~ should be left to stand at room temperature for 4–8 hours until the clot begins to contract, after which the blood is centrifuged at 1000 *g* for 10–15 minutes. Separated serum can be collected with a pipette and held at 4°C for a short period of time or stored at –20°C.

A minimum of 2 g of tissue from skin biopsies and organs should be collected for virus isolation and histopathology. Scabs from different locations of the body are also suitable for virus isolation and histopathology. They should be transported without additives in plain tubes. For the PCR, approximately 30–50 mg of tissue sample should be placed in a cryotube or similar container, kept at 4°C for transportation and stored at –20°C until processed. Tissue samples collected for virus isolation should be placed in a virus transport medium, such as Tris-buffered tryptose broth or minimal essential medium (MEM) without fetal calf serum (FCS), kept at 4°C for transportation and stored at –80°C until processed. Material for histology should be placed immediately after collection into ten times the sample volume of 10% formalin. The size of the samples should not exceed 0.5 cm × 1–2 cm. Samples in formalin can be transported at room temperature.

1. Detection of the agent

1.1. Transmission electron microscopy

TEM is a rapid method to demonstrate camelpox virus in scabs or tissue samples. ~~However,~~ A relatively high concentration of virus in the sample is required for positive diagnosis and camelpox virus cannot be differentiated from other *Orthopoxvirus* species. However, currently, TEM is the fastest method for distinguishing clinical cases of camelpox and orf respectively, although the poxvirus infections can be differentiated by serological techniques and by PCR (Mayer & Czerny, 1990).

1.1.1. Sample preparation

The size of a sample should be at least 30–50 mg. Mince the scabs or tissue sample with a disposable blade or sterile scissors and forceps. Grind the sample in a five-fold volume of isotonic phosphate-buffered saline (PBS), pH 7.0–7.4, with antibiotics (such as 10⁵ International Units [IU] penicillin and 10 mg streptomycin per ml) using a mortar and pestle with sterile sand. Transfer the sample into a centrifuge tube and freeze and thaw two to three times to release the virus from the cells. Vortex the samples while thawing. Place the tubes on ice and sonicate once for 30 seconds at 80 Hz. Centrifuge at 1000 *g* for 10 minutes to remove the gross particles and collect the supernatant (Pfeffer *et al.*, 1996; 1998b).

1.1.2. Test procedure

Place 10 µl of above-mentioned supernatant on poly-L-lysine-covered grids and incubate at room temperature for 5 minutes. Remove the fluid with a chromatography filter paper. Add one drop of 2% phosphotungstic acid (diluted in sterile water and pH adjusted to 7.2 with NaOH) to the grid, incubate at room temperature for 5 minutes and air dry. Examine the grid by TEM (Pfeffer *et al.*, 1996; 1998b).

Camelpox virus has a typical brick-shaped appearance with irregularly arranged, tubular surface proteins. Parapoxviruses are slightly smaller, ovoid-shaped and the surface proteins are regularly arranged.

1.2. Virus isolation in cell cultures

Camelpox virus can be propagated in a large variety of cell lines including Vero, MA-104 and MS monkey kidney, baby hamster kidney (BHK), and Dubai camel skin (Dubca) as well as in primary cell cultures (lamb testis, lamb kidney, camel embryonic kidney, calf kidney, and chicken embryo fibroblast) (Davies *et al.*, 1975; Tantawi *et al.*, 1974).

The samples are prepared for virus isolation as described above in Section B.1.1.1.

1.2.1. Test procedure

Incubate 400 µl of the supernatant for 1 hour at room temperature and then overnight at 4°C. Filter the supernatant through a 0.45 µm filter and inoculate into a 25 cm² flask of confluent cells. Flush the filter with 0.5 ml of the maintenance medium used in the cell culture and incubate the flasks at 37°C for 1 hour. Add 6–7 ml of fresh medium into the flask and continue the incubation for about 5–7 days. If there is any reason to suspect fungal contamination, the contaminated medium must be discarded and 5 µg/ml of amphotericin B added to a new medium. The flasks must be monitored daily for 5–7 days.

Characteristic, plaque-type cytopathic effect (CPE) showing foci of rounded cells, cell detachment, giant cell formation and syncytia may appear as soon as 24 hours post-inoculation. Syncytia may contain up to 20–25 nuclei (Tantawi *et al.*, 1974). The growth of camelpox virus in a cell culture can be confirmed by TEM, PCR or antigen-capture enzyme-linked immunosorbent assay (ELISA) (Johann & Czerny, 1993).

1.3. Virus isolation on chorioallantoic membrane of embryonated chicken eggs

Camelpox virus can be isolated on the chorioallantoic membrane (CAM) of 11- to 13-day-old embryonating chicken eggs. The eggs should be incubated at 37°C and after 5 days the eggs containing living embryos are opened and the CAM examined for the presence of characteristic pock lesions: dense, greyish-white pocks. Camelpox virus does not cause death in inoculated embryonated chicken eggs. The maximum temperature for the formation of pock lesions is 38.5°C. If the eggs are incubated at 34.5°C, the pocks are flatter and a haemorrhagic centre may develop (Tantawi *et al.*, 1974).

1.4. Immunohistochemistry

Immunohistochemistry for the detection of camelpox viral antigen is a relatively fast method and can be used instead of electron microscopy to establish a tentative diagnosis (Nothelfer *et al.*, 1995). Almost any polyclonal antibody against vaccinia virus is likely to produce reasonable results in this test because of the antigenic relatedness between vaccinia and camelpox viruses (Nothelfer *et al.*, 1995).

1.4.1. Test procedure

The following procedure for immunohistochemistry is described by Kinne *et al.* (1998) and Pfeffer *et al.* (1998b). The entire skin pustule should be collected for the immunohistochemical examination. Fix the tissue in 10% formalin, dehydrate through graded alcohols and embed in paraffin wax according to standard histopathological procedures. Cut approximately 3 µm sections and place on glass slides. Treat the deparaffinised and dehydrated sections with 3% H₂O₂, prepared in distilled water, for 5 minutes and wash with PBS. Incubate the slides for 60 minutes at 37°C with anti-vaccinia virus monoclonal antibody 5B4², diluted 1/500. Remove the monoclonal antibody by washing twice with cold PBS. Incubate the slides for 30 minutes with anti-mouse antibodies labelled with biotin. Wash with PBS for 5 minutes and incubate with streptavidin-peroxidase for 30 minutes. Wash again with PBS for 5 minutes and add diaminobenzidine as chromogen for 10 minutes. Examine the slides under the microscope using ×200–400 magnification for any brown-labelled pox antigen.

1.5. Molecular methods – detection of nucleic acids

~~1.5.1. Polymerase chain reaction~~

Extraction of nucleic acids

2 Monoclonal antibody 5B4 is available commercially. For further information, please contact the WOAHP Reference Laboratory: <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>.

Different kits and procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid is important and can be checked using a suitable method as appropriate to the circumstances.

1.5.1. Conventional PCR

Pathogen/ target gene	Primer (5'–3')	Concentration	Cycling parameters
<u>Method 1: Meyer <i>et al.</i>, 1994; GenBank Accession No.: NC_003391.1; amplicon size: 881 bp</u>			
<u>A type inclusion protein (ATIP)</u>	<u>Fwd: AAT-ACA-AGG-AGG-ATC-T- Rev: CTT-AAC-TTT-TTC-TTT-CTC</u>	<u>400 nM 400 nM</u>	<u>1 cycle of 94°C/5minutes; 29 cycles of: 94°C/1 min, 45°C/1 min; 72°C/2.5 min; and 72°C/10 min</u>

~~The PCR is a fast and sensitive method for the detection of orthopoxviral DNA. Several gel-based PCR methods have been described for the detection of camelpox viral DNA (Balamurugan *et al.*, 2009; Meyer *et al.*, 1994; 1997; Ropp *et al.*, 1995). A generic PCR assay, described by Meyer *et al.* (1994), allows the detection and differentiation of species of the genus *Orthopoxvirus* because of the size differences of the amplicons. Using the primer pair: 5'-AAT-ACA-AGG-AGG-ATC-T-3' and 5'-CTT-AAC-TTT-TTC-TTT-CTC-3', the gene sequence encoding the A type inclusion protein (ATIP) will be amplified. The size of the PCR product, specific for the camelpox virus, is 881 bp.~~

1.5.1.1. Conventional PCR test method ~~Test procedure for gel-based PCR~~

Suspend a small aliquot of crusted scabs in 90 µl of lysis solution (50 mM Tris/HCl, pH 8.0, 100 mM Na₂ EDTA, 100 mM NaCl, 1% sodium dodecyl sulphate) and add 10 µl of proteinase K (20 mg/ml). Digest the sample for 10 minutes at 37°C prior to the disruption of the scab or tissue with a microfuge tube pestle. Add another 350 µl lysis solution and 50 µl of proteinase K, mix gently and incubate for 3 hours at 37°C. Extract the lysed suspension with an equal volume of phenol/chloroform/isoamyl alcohol (25/24/1) and centrifuge at 8000 *g* at 4°C for 1 minute. Collect the upper aqueous phase and mix it again with an equal volume of phenol/chloroform/isoamyl alcohol (25/24/1). Centrifuge at 8000 *g* at 4°C for 1 minute and transfer the upper, aqueous phase to a new tube. Precipitate the DNA by adding 1/10 volume of 3 M sodium acetate and two volumes of ice-cold absolute ethanol. Place the mixture at –70°C for 30 minutes or –20°C overnight. Centrifuge at 15,000 *g* for 5 minutes at 4°C. Discard the supernatant and wash the pellet with 0.5 ml of 70% ethanol. Centrifuge at 15,000 *g* for 5 minutes. Discard the supernatant and air-dry the pellets. Resuspend the pellets in 10 µl of nuclease-free water. Alternatively, DNA can be extracted using commercially available DNA extraction kits.

DNA amplification is carried out in a final volume of 50 µl containing 2 µl of each dNTP (10 mM), 5 µl of 10 × PCR buffer, 1.5 µl of MgCl₂ (50 mM), 1 µl of each primer, 2.5 U *Taq* DNA polymerase, 1 µl DNA template and an appropriate volume of nuclease-free water.

~~Run the samples in a thermal cycler as follows: 5 minutes at 94°C; 1 minute at 94°C, 1 minute at 45°C, 2.5 minutes at 72°C. (20 cycles); 10 minutes at 72°C then hold at 4°C until analysis.~~

Mix 10 µl of a sample with loading dye solution and load in 1% agarose gel in TBE (Tris/Borate/EDTA) buffer containing ethidium bromide or cyanine nucleic acid stain. Load a parallel lane with a 100 bp DNA-marker ladder. Separate the products at 100 V for 30–40 minutes and visualise using an UV transilluminator. Confirm the positive reactions according to the amplicon size.

A commercial PCR kit has been developed that allows detection of *Orthopoxvirus* DNA and contains a second 'conventional' amplification system, consisting of primers to the haemagglutinin (HA) gene of the orthopoxvirus. The amplicon can be sequenced and identified by comparison with already existing orthopoxvirus sequences.

1.5.2.6 Real-time PCR

~~A small quantity of test material (blood, skin lesion, tissue) is suspended in 200 µl of lysis buffer and 20 µl of proteinase K and incubated at 65°C for 1 hour. The lysed sample is extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25/24/1) and centrifuged at 8000 *g* for 10 minutes. The upper aqueous phase is transferred to a clean tube and the DNA is precipitated by adding 1/10 volume 3 M sodium acetate and 2.5 volumes of ice-cold absolute ethanol. The~~

mixture is placed at –20°C for 1 hour followed by centrifugation at 13,000 g for 10 minutes. The supernatant is discarded and the pellet is washed with 0.5 ml 70% ethanol. The dried DNA pellet is resuspended in 30 µl of nuclease free water. Alternatively, DNA can be extracted using commercially available DNA extraction kits.

For standardisation of the PCR, extracted DNA from the purified virus is used to amplify a 166-bp product from the HA gene of the camelpox virus genome (Pfeffer et al., 1998b). PCR is performed in a 20-µl reaction containing 2 µl of 10× PCR reaction buffer, 1.6 µl of MgCl₂ (25 mM), 0.4 µl of dNTP (10 mM), (0.4 µl) of Taq polymerase, 5 units/µl, 2 µl DNA (approximately 50–100 ng), 6 pmol of each primer, and 2.5 pmol of each probe.

Pathogen/ target gene	Primer/probe (5'–3')	Concentration	Cycling parameters ^(a)
Method 1: Venkatesan <i>et al.</i> , 2012; Accession number NC_003391.1, amplicon size: 243 bp			
<u>ankyrin repeat protein (C18L) gene</u>	<u>CMLV C18L F: GCG-TTA-ACG-CGA-CGT-CGT-G</u> <u>CMLV C18L R: GAT-CGG-AGA-TAT-CAT-ACT-TTA-CTT-TAG</u> <u>CMLV C18L P: Cy5 CAT-CAA-TAC-AGC-CAT-CCA-GAA-AAG-TAG-BHQ-2</u>	<u>10 pmol/25 reaction</u> <u>10 pmol/25 reaction</u> <u>5 pmol/25 reaction</u>	<u>Initial denaturation: 1 cycle</u> <u>of 95°C/10 minutes;</u> <u>40 cycles of: denaturation:</u> <u>95°C/30 sec.</u> <u>annealing/elongation:</u> <u>60°C/1 min</u>

A positive result is indicated by an amplification that gives a cycle threshold (Ct) value of 37 or less.

2. Serological tests

All the viruses in the genus *Orthopoxvirus* cross-react serologically. However, within the genus only camelpox virus can cause pox-like lesions in camels. Parapox and camelpox viruses do not cross-react and so infections of camelpox and camel orf can be distinguished serologically. Most of the conventional serological tests are very time- and labour-consuming, which makes them unsuitable for primary diagnosis. However, serological tests are a valuable tool for secondary confirmatory testing and retrospective epidemiological studies in those areas where vaccination against camelpox is not practised.

2.1. Virus neutralisation test

In this method the test sera are titrated against a constant titre of camelpox virus (100 TCID₅₀ [50% tissue culture infectious dose]) on Vero cells.

2.1.1. Test procedure

- Sera are inactivated at 56°C for 30 minutes in a water bath.
- Sera are diluted in a twofold dilution series across the 96-well, flat-bottomed, cell-culture grade microtitre plate in serum-free cell culture medium (25 µl volumes). Individual serum controls, together with negative and known positive control sera, must also be included in each test.
- A dilution of stock virus made up to contain 100 TCID₅₀ per 25 µl is prepared using serum-free cell culture medium containing antibiotics.
- 25 µl of the appropriate stock virus dilution is added to every well containing 25 µl of each serum dilution, except the test serum control wells and cell control wells on each plate.
- Plates are covered and incubated 1 hour at 37°C in 5% CO₂ atmosphere.
- A cell suspension is made from 3–4 days old Vero cells using a concentration that will ensure confluent monolayers in the microtitre plate wells within 18–24 hours after seeding.
- A volume of 100 µl of cell suspension is added to every well, the plates are sealed with tape and incubated at 37°C in 5% CO₂ atmosphere for 3–4 days.
- Plates are examined microscopically for CPE and results are recorded on a work sheet. Wells are scored as positive for neutralisation of virus if 100% of the cell monolayer is intact. The highest dilution of serum resulting in complete neutralisation of virus (no CPE) in half of the test wells is the 50% end-point titre of that serum. If necessary, the titre can be determined by the Spearman–Kärber method. A titre of 1/8 or greater is considered to be positive.

2.2. Enzyme-linked immunosorbent assay for the detection of antibodies against camelpox virus

The following procedure for the antibody ELISA for *Camelpox virus* is described by Azwai *et al.* (1996) and Pfeffer *et al.* (1998b). The following description gives general guidelines for the test procedure.

2.2.1. Preparation of the antigen

- i) Harvest the cell culture when 100% infected with camelpox virus. Freeze and thaw two to three times. Sonicate for 30 seconds at 80 Hz on ice to release the virus from the cells.
- ii) Centrifuge at 1000 *g* for 10 minutes and collect the supernatant.
- iii) Centrifuge the supernatant at 45,000 *g* at 4°C for 1 hour. Re-suspend the pellet in PBS.
- iv) Add NaCl to a final concentration of 330 mM and polyethylene glycol (PEG 6000) to a final concentration of 7%.
- v) Stir overnight at 4°C, centrifuge at 3000 *g* at 4°C for 10 minutes and wash the pellet twice with 15 mM NaCl.
- vi) Freeze and thaw, and treat with 1% non-ionic detergent (Nonidet P40, Sigma) at 37°C for 3 hours.
- vii) Freeze and thaw and centrifuge at 3000 *g* for 10 minutes at 4°C.
- viii) Collect the supernatant and dialyse at least three times against PBS.
- ix) Measure the protein concentration as described by Lowry *et al.* (1951).
- x) Store the aliquots at –20°C.

2.2.2. Preparation of rabbit anti-camel IgG horseradish-peroxidase conjugate

Rabbit anti-llama and anti-camel IgG horseradish-peroxidase conjugates are commercially available. The method for producing monoclonal antibodies for camel IgM and IgG has been described by Azwai *et al.* (1995). However, rabbit anti-camel IgG horseradish-peroxidase can be replaced with a commercially available protein A-peroxidase, *Staphylococcus aureus*/horseradish conjugate.

- i) Precipitate camel sera twice adding saturated ammonium sulphate to a final concentration of 40% (v/v) (29.6% ammonium sulphate [w/v]) at room temperature. Centrifuge at 12,000 *g* for 15 minutes and dissolve in PBS, pH 7.2. Dialyse against several changes of PBS overnight.
- ii) Separate the immunoglobulins using gel filtration chromatography: a column (2.6 × 100 cm) can be used to separate the salt precipitated immunoglobulins (IgM and IgG) by size. Elution can be effected with PBS at 20 ml/hour and 6 ml fractions can be collected. Determine the protein concentrations by absorbance at 280 nm.

2.2.3. Antiserum production

Immunise rabbits with a subcutaneous injection of camel IgG emulsified in appropriate adjuvant. The animals should be immunised three times to booster antibody production. Collect the serum and store at –20°C until used.

2.2.4. Test procedure

- i) Coat 96-well microtitre ELISA plates with prepared antigen at 1 µg/ml in carbonate/bicarbonate buffer, 0.05 M, pH 9.6 (100 µl per well).
- ii) Incubate the ELISA plates in a humid chamber (100% humidity) at 37°C for 1 hour and then overnight at 4°C.
- iii) Wash off the unbound antigen with PBS containing 0.05% Tween 20 (PBS/Tween) three times.
- iv) Add 100 µl of test and control serum at a predetermined optimal dilution in blocking buffer (PBS containing 0.05% Tween 20, and 1% fat-free milk powder) in duplicate wells.
- v) Incubate the plates for 30 minutes at 37°C.
- vi) Wash the plates three times with PBS/Tween.

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- vii) Dilute rabbit anti-camel IgG horseradish-peroxidase conjugate or protein A-peroxidase, *Staphylococcus aureus*/horseradish conjugate at a predetermined working dilution in blocking buffer and add 100 µl into the wells.
 - viii) Incubate the plates at 37°C for 30 minutes.
 - ix) Wash the plates three times with PBS/Tween.
 - x) Make the reaction visible using 100 µl per well of chromogen 3,3',5,5'-tetramethylbenzidine (TMB) with hydrogen peroxide and incubate 15 minutes at 37°C with shaking.
 - xi) Stop the reaction 10 minutes later by 2 M H₂SO₄ at a volume of 50 µl/well.
 - xii) Measure the values with a photometer at a wavelength of 450 nm. Seropositivity can be calculated as values above the mean +2 standard deviations from negative control sera.

C. REQUIREMENTS FOR VACCINES

C1. Live attenuated vaccine

1. Background

1.1. Rationale and intended use of the product

Currently live attenuated and inactivated vaccines are commercially available. The live attenuated vaccine was prepared from a strain isolated from a dromedary camel calf that had generalised camelpox (Wernery, 2000). The live attenuated vaccine gives long-term protection against camelpox (Wernery & Zachariah, 1999). However, a booster vaccination is recommended for young animals at the age of 8–12 months, 2–3 months after the initial vaccination, to avoid interference by maternal antibodies. When inactivated vaccine is used, the animals must be vaccinated annually.

Guidelines for the production of the veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*.

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

Samples (such as crusty material from the nose, skin lesions or scabs) are collected from a camel calf showing generalised camelpox lesions. The samples are crushed in MEM with antibiotics, centrifuged, sterile-filtered and inoculated onto confluent Vero cells and on fetal camel skin cell line (Dubca). CPE is observed after 4 days of incubation at 37°C. When 80% of the cells are infected, cell culture is harvested, frozen and thawed. This procedure is done three times followed by sonication at 80 Hz on ice to release the virus. The suspension is clarified by centrifugation at 1000 **g** for 10 minutes. The supernatant is collected, and the identity of camelpox virus is confirmed using different test methods. After the 10th passage on Vero cells, a plaque test is carried out and the bigger plaque is chosen for the vaccine preparation. The plaque-purified virus is then passaged 110 times on Vero cells to attenuate the virus and designated as the master seed virus (MSV). The MSV is kept freeze dried and frozen at –80°C.

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

The purity and identity of the seed virus and the cells used for the vaccine production is proved. The seed virus is demonstrated free from contamination with adventitious viruses, bacteria, fungi or mycoplasma.

2.2. Method of manufacture

2.2.1. Procedure

The cells used for the production of vaccine preparation are prepared using a seed-lot system. The seed virus is cultured on Vero cells. When the cell monolayer is confluent, the cells are infected with the vaccine virus. Cell culture is harvested when the cells are 100% infected with camelpox virus. The supernatant is clarified, mixed with a stabiliser, bottled and freeze-dried.

2.2.2. Requirements for ingredients

Cell cultures and all animal origin products used in the production and maintenance of cells are proved free of adventitious viruses, bacteria, fungi and mycoplasma.

2.2.3. In-process controls

CPE is checked during cultivation of working virus seed. Uninoculated control cells should retain their morphology until the time of harvesting. Virus multiplication is demonstrated by titration with the harvested supernatant.

2.2.4. Final product batch tests

i) Sterility/purity

The procedure for testing for sterility and freedom from contamination of biological materials intended for veterinary use is described in chapter 1.1.9.

ii) Safety

Using the recommended route of administration, each batch of vaccine is tested in ten naive camels, using ten times the recommended dose per animal. The animals are observed for 7–14 days for any adverse reactions.

iii) Batch potency

The amount of virus present in the live attenuated vaccine is titrated on cell culture and the end titre is calculated.

2.3. Requirements for regulatory approval

2.3.1. Safety requirements

i) Target and non-target animal safety

Live attenuated camelpox vaccine causes no clinical signs in camelpox-susceptible Old World camels and New World camels. Less than 1% of animals show a rise in temperature by a maximum of 1°C.

ii) Reversion-to-virulence

There are no reports of reversion to virulence by the live attenuated vaccine.

iii) Precautions

There is a low risk of human infection as camelpox is host specific. However, self-inoculation may lead to zoonotic camelpox virus infection with a vaccine virus.

2.3.2. Efficacy

Efficacy in susceptible animals is demonstrated in naive dromedaries. The experimental animals should be vaccinated twice with the live attenuated vaccine and 3 weeks after the last vaccination the camels should be challenged with a virulent camelpox field strain. The virulence of the challenge virus should be demonstrated by inoculation of the virus into unvaccinated control animals. The vaccinated animals should not show any clinical signs whereas the unvaccinated group should develop characteristic clinical signs of camelpox. The long-term immunity provided by the live attenuated vaccine can also be confirmed by challenging the vaccinated animals 6 years later. The efficacy of the live attenuated vaccine should be further evaluated by measuring the antibody levels against camelpox virus 21–30 days after vaccination using ELISA and virus neutralisation test.

The live attenuated vaccine can protect dromedaries from an infection for at least 6 years, but may be lifelong.

2.3.3. Stability

Vaccines should be stored at 4–8°C, with minimal exposure to light. The shelf life is determined by virus titration.

C2. Inactivated vaccine

1. Background

1.1. Rationale and intended use of the product

An inactivated vaccine has been available since 1992 and used mainly in North Africa to prevent the disease (El Harrak & Loutfi, 1999). The inactivated vaccine is prepared from a strain isolated during the 1984 outbreak in Morocco from a dromedary camel presenting with generalised camelpox (El Harrak *et al.*, 1991). The inactivated vaccine gives good protection against camelpox after a double injection administered at a 3- to 6-month interval followed by the annual booster. The vaccine is recommended from the age of 8–12 months to avoid interference by maternal antibodies.

Guidelines for the production of the veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The production of commercially available inactivated camelpox vaccine is described below.

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

Pox lesions from an adult dromedary camel presenting generalised camelpox infection are removed, crushed in PBS with antibiotics, centrifuged, sterile-filtered and inoculated onto the chorioallantoic membrane of specific pathogen free eggs for the first isolation (El Harrak *et al.*, 1991). The pox lesions obtained are passed on confluent Vero cells serially seven times. A CPE is routinely observed after 3–4 days of incubation at 35°C. When 80% of the cells are infected, viral suspension is harvested, frozen and thawed. This procedure is done twice to release the virus. The suspension is collected and the identity of the camelpox virus is proven using different test methods. The virus at its eighth passage level was designated the master seed virus (MSV) and named Laayoune T8 strain. The MSV is kept frozen at –80°C.

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

The purity, identity and titre of the seed virus as well as the purity of the Vero cells used for the vaccine production is proved. The seed virus is demonstrated free from contamination with adventitious viruses, bacteria, fungi or mycoplasma.

2.1.3. Validation as a vaccine strain)

Laayoune T8 strain was validated as a vaccine strain after three successive passages on Vero cells using the limiting dilution method to purify the virus. CPE characteristics and the virus titre are reproducible and freedom from extraneous agents proven.

2.2. Method of manufacture

2.2.1. Procedure

The seed virus is grown on Vero cells. Cells used for vaccine production are prepared using a seed-lot system. The infection with the seed virus takes place on confluent Vero cells in roller bottles or in bio-generators with micro-carriers. Viral suspension is harvested when the cells are 80% infected with camelpox virus. The virus is inactivated using betapropiolactone then mixed with aluminium hydroxide as an adjuvant and bottled.

2.2.2. Requirements for ingredients

The seed virus, cell cultures and all animal origin ingredients used in the production are proved free of adventitious viruses, bacteria, fungi and mycoplasma.

2.2.3. In-process controls

Cell sterility and purity are tested during the production process. The harvested virus is tested for sterility and infectious titre, the inactivated antigen is also checked for sterility and complete inactivation.

2.2.4. Final product batch tests

i) Sterility/purity

Tests for sterility and freedom from contamination of biological materials intended for veterinary use is found in chapter 1.1.9.

ii) Identity

Identity of the vaccine virus is confirmed by PCR on the inactivated final product.

iii) Safety

Using the recommended route of administration, each batch of vaccine is tested in two naïve camels, using two times the recommended field dose per animal. The animals are observed for 14 days for any adverse reactions.

iv) Batch potency

The amount of virus present in the inactivated vaccine is measured by titration on cell culture before inactivation and by real time PCR carried out on antigen before addition of the adjuvant.

2.3. Requirements for regulatory approval

2.3.1. Manufacturing process

The inactivated camelpox vaccine is produced according to good manufacturing practices (GMP) standards and procedures recommended for inactivated veterinary vaccines.

2.3.2. Safety requirements

i) Target and non-target animal safety

Inactivated camelpox vaccine causes no clinical signs or rise in body temperature when administered to camelpox-susceptible animals. An inflammatory reaction due to the presence of the adjuvant may appear at the injection site with no influence on the animal's health.

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

Not applicable for inactivated vaccines.

iii) Precautions (hazards)

There are no precautions necessary as camelpox is host specific.

2.3.3. Efficacy requirements

Efficacy is demonstrated in six naïve calf dromedaries challenged with a virulent field strain after two injections with the inactivated vaccine at a 3-month interval. The virulent strain is titrated on the animal skin comparatively on four vaccinated and two unvaccinated control animals. The obtained infectious titre on vaccinated animals is at least 1.5 log ID₅₀ less than the titre obtained on control animals. In addition, vaccinated animals do not show any signs of generalised disease. The efficacy of the inactivated vaccine was also proven by the development of antibodies after vaccination that were evaluated by ELISA and the virus neutralisation test (El Harrak & Loutfi, 1999).

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

There is no vaccine available permitting a DIVA strategy for camelpox control.

2.3.5. Duration of immunity

The long-term immunity caused by the inactivated vaccine was also confirmed by challenging the vaccinated animals 1 year after the primary vaccination. Duration of the conferred immunity with inactivated vaccine lasts for a minimum of 1 year after double injection of the vaccine, as tested on young naïve dromedary camels. After multiple vaccinations, immunity may be longer in adult dromedaries; no vaccination failures reported in the field with animals not regularly vaccinated.

2.3.6. Stability

Vaccines should be stored at 4–8°C, with minimal exposure to light. The shelf life is 24 months.

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* *

NB: There is a WOAH Reference Laboratory for camelpox (please consult the WOAH Web site: <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>).

Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for camelpox

NB: FIRST ADOPTED IN 2008. MOST RECENT UPDATES ADOPTED IN 2021.

Appendix 1

Disease name: Camelpox

Intended purpose of test: population freedom from infection

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>ELISA</u> ☒ ☒ ☒	<u>Serum</u>	<u>N/A</u>	<u>N/A</u>	<u>None</u>	<u>Fast result within 24 hours</u>	<u>ELISA kits are not commercially available</u>	<u>Azwai <i>et al.</i> (1996)</u>
<u>Virus neutralisation</u> ☒ ☒ ☒	<u>Serum</u>	<u>Dsp: 100%</u> <u>Dse: 100%</u>	<u>N/A</u>	<u>None</u>		<u>Time and labour consuming. Needs live virus and special cell lines</u>	<u>Not available</u>

Appendix 2

Disease name: Camelpox

Intended purpose of test: individual animal freedom from infection prior to movement

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>ELISA</u> ☒ ☐ ☐	<u>Serum</u>	<u>N/A</u>	<u>N/A</u>	<u>None</u>	<u>Fast result within 24 hours</u>	<u>ELISA kits are not commercially available</u>	<u>Azwai <i>et al.</i> (1996)</u>
<u>Virus neutralisation</u> ☒ ☐ ☐	<u>Serum</u>	<u>Dsp: 100%</u> <u>Dse: 100%</u>	<u>N/A</u>	<u>None</u>		<u>Time and labour consuming. Needs live virus and special cell lines</u>	<u>Not available</u>

Appendix 3

Disease name: Camelpox

Intended purpose of test: confirmation of clinical cases

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>TEM</u> ☒ ☒ ☒	<u>Scabs</u> <u>Lung</u>	<u>Dsp: 100%</u> <u>Dse: 90%</u>	=	<u>None</u>	<u>Dsp: 100%</u> <u>DSe: 90%</u>	<u>Needs specialised laboratory, rarely available in countries where camel pox exist</u>	<u>Pfeffer <i>et al.</i> (1998b)</u>
<u>Virus isolation</u> ☒ ☒ ☒	<u>Scabs</u> <u>Lung</u>	<u>Dsp: 100%</u> <u>Dse: 100%</u>	=	<u>None</u>	<u>Dsp: 100%</u> <u>DSe: 100%</u>	<u>Needs specialised laboratory and special cell lines. Takes several days for the diagnosis. The isolated virus must be confirmed by other tests</u>	<u>Pfeffer <i>et al.</i> (1998b)</u>
<u>Immuno-histochemistry</u> ☒ ☒ ☒	<u>Scabs</u> <u>Lung</u>	<u>Dsp: 99.7%</u>	=	<u>None</u>	<u>Dsp: 99.7%</u> <u>Embedded tissue blocks can be investigated years after they have been made</u>	<u>Needs specialised laboratory. Immunohistochemistry cannot differentiate OPV members unless specific monoclonal antibodies are used</u>	<u>Czerny <i>et al.</i> (1989)</u> <u>Kline <i>et al.</i> (1998)</u>
<u>PCR</u> ☒ ☒ ☒	<u>Scabs</u> <u>Lung</u> <u>Virus</u>	<u>Dsp: 100%</u> <u>Dse: 100%</u>	=	<u>Yes</u>	<u>Best diagnostic test</u> <u>Fast result within 24 hours</u>	<u>Needs specialised laboratory.</u>	<u>Pfeffer <i>et al.</i> (1998b)</u> <u>Meyer <i>et al.</i> (1994)</u>
<u>ELISA</u> ☒ ☐ ☐	<u>Serum</u>	<u>N/A</u>	<u>N/A</u>	<u>None</u>	<u>Fast result within 24 hours</u>	<u>ELISA kits are not commercially available</u>	<u>Azwai <i>et al.</i> (1996)</u>
<u>Virus neutralisation</u> ☒ ☐ ☐	<u>Serum</u>	<u>Dsp: 100%</u> <u>Dse: 100%</u>	<u>N/A</u>	<u>None</u>		<u>Time and labour consuming. Needs live virus and special cell lines</u>	=

Appendix 4

Disease name: Camelpox

Intended purpose of test: prevalence of infection – surveillance

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>ELISA</u> <u>✓✓✓</u>	<u>Serum</u>	<u>N/A</u>	<u>N/A</u>	<u>None</u>	<u>Fast result within 24 hours</u>	<u>ELISA kits are not commercially available</u>	<u>Azwai <i>et al.</i> (1996)</u>
<u>Virus neutralisation</u> <u>✓✓✓</u>	<u>Serum</u>	<u>Dsp: 100%</u> <u>Dse: 100%</u>	<u>N/A</u>	<u>None</u>		<u>Time and labour consuming. Needs live virus and special cell lines</u>	<u>Not available</u>

Appendix 5

Disease name: Camelpox

Intended purpose of test: immune status in individual animals or populations post-vaccination

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>ELISA</u> <u>✓✓✓</u>	<u>Serum</u>	<u>N/A</u>	<u>N/A</u>	<u>None</u>	<u>Fast result within 24 hours</u>	<u>ELISA kits are not commercially available</u>	<u>Azwai <i>et al.</i> (1996)</u>
<u>Virus neutralisation</u> <u>✓✓✓</u>	<u>Serum</u>	<u>Dsp: 100%</u> <u>Dse: 100%</u>	<u>N/A</u>	<u>None</u>		<u>Time and labour consuming. Needs live virus and special cell lines</u>	<u>Not available</u>

MEETING OF THE BIOLOGICAL STANDARDS COMMISSION

Paris, 8–12 September 2025

CHAPTER 3.6.4.

EQUINE ENCEPHALOMYELITIS
(EASTERN, WESTERN AND VENEZUELAN)

SUMMARY

Description and importance of the disease: Equine encephalomyelitis (Eastern, Western and Venezuelan) are zoonotic diseases caused by arthropod-borne viruses of the family Togaviridae, genus Alphavirus: Alphavirus eastern (EEEV), Alphavirus western (WEEV) and Alphavirus venezuelan (VEEV), distributed in the Americas. Eastern, Western and Venezuelan equine encephalomyelitis (EEE, WEE and VEE), viruses belong to the genus Alphavirus of the family Togaviridae. EEE, WEE and VEE are alphaviruses present in the Americas and can cause disease in both humans and equids with encephalitis in most clinical cases. Although closely related, they are genetically and antigenically distinct. All three viruses can cause severe or fatal encephalitis in humans and equids, with differences in epidemiology, virulence, and geographical distribution. EEEV, and WEEV, and VEE are typically primarily maintained in complex life cycles involving nature by alternating between vertebrate hosts and vector mosquitoes. Different mosquito species (Culex spp., Aedes spp., etc.) may be involved in the transmission cycle in different vertebrate hosts depending on geographical and ecological locations. Vertebrate hosts, principally wild birds, but also rodents, reptiles and lagomorphs, can develop viraemic infection, playing an important role as reservoirs in the enzootic transmission of these viruses. Horses, as humans, are incidental dead-end hosts for EEEV and WEEV and thus, not important in disease transmission. Moreover, horses are considered sentinel animals, as they are the primary animal host to show clinical signs, allowing authorities to be alerted and minimise the potential impact on public health. The VEEV complex includes epizootic (I-AB, I-C) and enzootic (I-D, I-E, I-F, II to VI) subtypes. Epizootic strains can cause widespread equine and human outbreaks, within a cycle between equids and mosquitoes. In contrast, enzootic strains circulate in rodent–mosquito cycles (except for Tonate virus) and occasionally infect humans and horses. Encephalitis caused by these viruses may occur sporadically in horses and humans from mid-summer to late autumn in temperate regions but can occur year-round in tropical regions, depending on climate conditions that support the presence of the mosquito vector. Mosquitoes' seasonal activity and interactions with host and viruses is strongly influenced by ecological conditions such as temperature, precipitation and relative humidity. Thus, warmer and wetter weather attributed to climate change can directly affect the presentation of clinical disease during the year. The incubation period in horses for EEE or WEE is 5–14 days while for VEE it is 7 days, although neurological signs may appear around day 5. Clinical disease in horses is characterised by fever, anorexia, and severe depression. In severe cases, it can progress to hyperexcitability, blindness, ataxia, severe mental depression, recumbency, convulsions, and death. The disease, which is commonly known as sleeping sickness, includes clinical signs such as fever, anorexia, depression, and neurological disorders leading to high mortality, particularly for EEEV and VEEV.

In humans, EEEV causes the most severe illness, with mortality rates of 30–70% and frequent neurological sequelae. WEEV infections are generally milder, while VEEV produces flu-like or

neurological disease in both humans and equids during epizootics. Other mammalian species such as cattle, sheep, pigs, deer, and dogs may also be affected, and various bird species, including game birds and ratites, can suffer high mortality.

EEEV is found in North and South America, with the South American strain now classified as Madariaga virus. WEEV historically circulated across the Americas but has not caused major outbreaks since 1999, although re-emergence events have been reported in South America in 2023-2024. Epizootic VEEV have been always identified in Central and northern South America, with a dissemination event in 1971 to southern USA, while enzootic cycles of VEEV subtypes occur from southern USA (Everglade virus) to northern Argentina (Rio Negro virus).

EEE virus infection in horses is often fatal, while WEE virus can cause a subclinical or mild disease with less than 30% mortality. The principal reservoir hosts for EEE and WEE are passerine birds. Most infections in birds are nonclinical, but EEE and WEE viruses have been reported to cause disease in poultry, game birds and ratites. Small mammals, such as rodents may also amplify EEE. Sporadic cases of EEE, WEE, and VEEV have been reported in cows, sheep, pigs, deer, emus and dogs. Horses, and humans, are incidental dead-end hosts for EEE and WEE. However, some horses may develop a transient viraemia that has been suggested to be sufficient to transmit EEE virus to mosquitoes under the right conditions.

VEE viruses are considered to be one of the most important equine pathogens in México, South and Central America. For example, one epidemic in Colombia associated with up to 100,000 equid deaths and 250,000 human cases. In contrast to EEE and WEE, horses play a key role in virus amplification, disease spread and maintenance of epizootics of VEE. The VEE complex of viruses includes six antigenic subtypes (I-VI). Within subtype I there are five antigenic variants (variants AB-F). Originally, subtypes I-A and I-B were considered to be distinct variants, but they are now considered to be identical (I-AB). Antigenic variants I-AB and I-C are associated with epizootic activity in equids and humans. The other three variants of subtype I (I-D, I-E, I-F) and the other five subtypes of the VEE (II-VI) circulate in natural enzootic cycles. Equidae are not dead-end hosts for VEE virus; they serve as amplifying hosts for epizootic VEE strains while enzootic VEE viruses cycle primarily in sylvatic rodents and mosquitoes. Enzootic variants and subtypes have been considered to be non-pathogenic for equids but can cause clinical disease in humans. During 1993 and 1996, limited outbreaks of encephalitis in horses in Mexico were shown to be caused by enzootic VEEV viruses of subtype IE. More recently, sporadic outbreaks have occurred in Mexico, Central America, and northern and western parts of South America. Human enzootic subtypes extend more broadly into northern Central America and South America (Weaver et al., 2012).

Detection of the agent: A presumptive diagnosis of EEE, WEE or VEE can be made when susceptible horses display the characteristic somnolence, fever and other signs of neurological disease in endemic areas where haematophagous insects are active. Detection of equine encephalitis viruses (EEEV, WEEV, and VEEV) relies on virus isolation and molecular assays with sensitivity and biosafety requirements varying among them. Virus isolation remains the definitive diagnostic method and is performed by inoculating susceptible cell cultures. There are no characteristic gross lesions. Histopathological lesions can provide a presumptive diagnosis; EEE virus can usually be isolated from the brain and sometimes other tissues of dead horses, however WEE and VEE viruses are rarely isolated. The viruses can be isolated from field specimens including central nervous system (CNS) and organs, especially liver and spleen, by inoculating embryonated chicken eggs or cell cultures. Molecular methods provide rapid and specific detection and are now considered the most practical approach for diagnosis. The virus genome can be identified by reverse-transcription (RT)-nested polymerase chain reaction (PCR), which is currently the method of choice for alphaviruses detection. reverse-transcription polymerase chain reaction (RT-PCR), complement fixation (CF), immunofluorescence, or plaque reduction neutralisation (PRN) tests. Specific identification of epizootic Characterisation of VEEV virus variants subtypes, which is critical to differentiate enzootic and epizootics strains, can be made by whole gene sequencing the indirect fluorescent antibody test, or a differential PRN test using subtype or variant-specific monoclonal antibody, or by nucleic acid sequencing.

Serological tests: Antibodies can be identified by PRNT test, haemagglutination inhibition (HI), CF, or IgM capture enzyme-linked immunosorbent assay or virus-neutralisation test (VNT), which remain the gold standard methods due to their high specificity and ability to differentiate between infections; however, they require live virus and must be conducted under appropriate biosafety containment.

Requirements for vaccines: *EEE and WEE vaccines for equines are safe and generate an antibody response that ensures protection-immunogenic, requiring two initial doses, followed by one up to twice a year boosters in high-risk areas. For VEEV, vaccination in horses relies on preparations derived from the TC-83 strain as a live-attenuated in endemic regions. The attenuated vaccine is immunogenic when administered intramuscularly but may cause adverse reactions in a proportion of recipients and shows variable immune responses. The only recommended vaccines against VEE are an live attenuated virus vaccine, made with strain TC-83, or inactivated virus preparations also made from this strain. Attenuated virus is immunogenic when given by intramuscular injection and can cause adverse reactions in the recipient.*

Formalin-inactivated virulent VEE virus preparations should not be used in equids, as residual virulent virus can remain after formalin treatment, causing severe illness in both animals and humans. Epizootics of VEE have occurred from the use of such formalin-treated viruses.

A. INTRODUCTION

1. Description of the disease and aetiology: general information

Eastern equine encephalomyelitis (EEE), Western equine encephalomyelitis (WEE) and Venezuelan equine encephalomyelitis (VEE) viruses—*Alphavirus eastern* (EEEV), *Alphavirus western* (WEEV) and *Alphavirus venezuelan* (VEEV) are members of the genus *Alphavirus* of the family *Togaviridae* and are distributed in the Americas (Arechiga-Ceballos & Aguilar-Setien, 2015). Although closely related, EEEV, WEEV and VEEV are genetically and antigenically distinct (Arechiga-Ceballos & Aguilar-Setien, 2015; Kumar *et al.*, 2018). These alphaviruses are all capable of causing lethal encephalitis in humans and horses; however, the patterns of disease, disease severity, and incidence vary greatly. The natural ecology for virus maintenance typically occurs via alternating infection of birds and mosquitoes, mosquitoes (EEE and WEE), mosquitoes and rodents (VEE enzootic cycle), or mosquitoes and horses (only for epizootic VEEV epizootic cycle subtypes). EEE virus has also been isolated from snakes, and these may have a role as reservoir hosts (Bingham *et al.*, 2012; White *et al.*, 2011). Clinical disease may be observed in humans and horses, both of which are incidental dead-end hosts for EEEV and WEEV and thus not important in disease transmission (Arechiga-Ceballos & Aguilar-Setien, 2015; Go *et al.*, 2014; Kumar *et al.*, 2018; Salimi *et al.*, 2016; Zacks & Paessler, 2010). However some horses may develop a transient viraemia that has been suggested as potentially sufficient to transmit EEE virus to mosquitoes under the right conditions (Franklin, 2002).

All three viruses are classified as alphaviruses present in the Americas and are present in the Americas (Arechiga-Ceballos & Aguilar-Setien, 2015). EEE has lineages associated with both North America (predominately the central and eastern United States of America [USA], Canada, and Central America) and South America. The South American strains of EEE, previously known as lineages II, III, and IV, are now known as Madariaga virus (Arrigo *et al.*, 2010) after strains causing outbreaks in Darien, Panama in 2010 (Carrera *et al.*, 2013) led to the re-evaluation of viral sequencing and reclassification of the South American strain of EEE (Madariaga virus) as a separate divergent strain. WEE was historically detected primarily in the western USA and Canada, Mexico, and Central and South America (Arechiga-Ceballos & Aguilar-Setien, 2015; Kumar, 2018; Morris, 1989; Reisen & Monath, 1989; Walton, 1981). Highlands J virus, antigenically related to WEE virus, has been isolated in the eastern USA. Although Highlands J virus is generally believed not to cause disease in mammals, it has been isolated from the brain of a horse in Florida dying of encephalitis (Karabatsos *et al.*, 1988). Epizootic VEE viruses have been identified in Central and South America with the most recent spread into Southern USA from Mexico in 1971 (Kumar, 2018; Oberste *et al.*, 1999; Salimi *et al.*, 2016; Snider *et al.*, 1993; Wang *et al.*, 1999; Weaver *et al.*, 1999; Zehmer *et al.*, 1974).

In equids, The clinical signs of EEE, WEE and VEE can be identical. The disease caused by any of the three viruses is also known as sleeping sickness. Following the incubation period of (1–14 days, depending on the virus and strain for EEE and WEE, 7 days for VEE, although neurological signs may appear around day 5), clinical signs appear and include fever, anorexia, and depression. A presumptive diagnosis of equine viral encephalomyelitis in unvaccinated horses can be made if the characteristic somnolence is neurological signs and fever are observed during the summer in temperate climates or the wet season in tropical and subtropical climates, when the mosquito vector is plentiful. However, a number of other diseases, such as West Nile virus (WNV) (Chapter 3.1.26), rabies (Chapter 3.1.19), neurological equid herpesvirus 1 infection (equine rhinopneumonitis) (Chapter 3.6.8) and other infectious, parasitic, or non-infectious agents can produce similar clinical signs, and the diagnosis must be confirmed by the described diagnostic test methods.

EEE virus causes severe disease in humans with a mortality rate of 30–70% and a high frequency of permanent neurological sequelae in patients who survive. The North American variant is considered to be more pathogenic than South American strains (Madariaga virus) (Kumar *et al.*, 2018). EEE virus has been reported to cause disease

in mammals other than equines and humans, including cows (McGee *et al.*, 1992; Pursell *et al.*, 1976), sheep (Bauer *et al.*, 2005), pigs (Elvinger *et al.*, 1996), white-tailed deer (Tate *et al.*, 2005), and dogs (Farrar *et al.*, 2005). EEE virus infections are usually observed in limited geographical areas. WEE is usually mild in adult humans, but can be a severe disease in children. The fatality rate is between 3 and 14%. WEE virus infection in horses has historically been observed over a wide geographical area, e.g. sporadic cases over 2590 km² (1000 square miles); however, no disease outbreaks attributed to WEE have occurred since 1999.

Most encephalomyelitis infections in domestic fowl are caused by EEE virus and occur in the east coast states of the USA. Isolated events of high mortality in captive-raised game birds, primarily pheasants, chukars, aquarium penguins, and quail have been traced to EEE, WEE, or Highlands J virus infection (Morris, 1989; Reisen & Monath, 1989; Tuttle *et al.*, 2005). Although virus is introduced by mosquitoes, transmission within the flocks is primarily by feather-picking and cannibalism (Kumar *et al.*, 2018). Both EEE and WEE viruses have caused fatal disease in ratites. Haemorrhagic enteritis has been observed in emus infected with EEE and WEE viruses, and morbidity and mortality rates may be greater than 85%. EEE and Highlands J viruses have been found to produce depression, somnolence, decreased egg production, and increased mortality in turkeys (Guy, 1997).

1.1. EEEV and WEEV complex

EEEV was first isolated in horses in 1933 in an outbreak in Delaware, New Jersey and Virginia (United States of America [USA]). Over the years, the disease has spread extensively from south-eastern Canada to south-eastern USA; the largest EEE outbreak in the USA was reported in 2019, primarily in the northeastern states, affecting both horses and humans (Hill *et al.*, 2023). Among the EEEV complex, species present in North America (NA-EEEV) are antigenically and genetically different from those present in South America (SA-EEEV); they also differ in their pathogenicity in equids and humans (Go *et al.*, 2014) (Table 1). These differences between NA-EEEV and SA-EEEV may reflect ecological variations in their enzootic transmission cycle.

Birds are thought to be the principal reservoir hosts for EEEV, though rodents and other small mammals might also amplify this virus. In North America, NA-EEEV strains are maintained between passerine birds as reservoir/amplification host and *Culisetta melanura*, as the main enzootic vector in swamp habitats. Other vectors, such as *C. peccator*, *C. erraticus* and *Uranotaenia sapphirine*, have also been described as feeding on reptiles and amphibians. Bridge vectors, such as *Aedes* and *Coquillettidia* spp., are known to transmit the virus to humans and equines. Passerine birds develop extremely high levels of viremia, enough to infect both enzootic vectors as well as a variety of bridge vectors. In pheasants, NA-EEEV is transmitted through feather picking and cannibalism.

In South America, the primary reservoir hosts for SA-EEEV strains, known also as Madariaga virus (MADV), are still uncertain, though small mammals such as rodents and marsupials are thought to play an important role in the enzootic cycle together with *Culex (melanoconion)* subgenus as the principal vector (Go *et al.*, 2014; USDA, 2024).

For both NA-EEEV and SA-EEEV, humans and equids are dead-end hosts as they do not develop sufficient viremia to transmit the virus, and during outbreaks or epidemics, horses tend to be the first to develop clinical signs and often serve as an indicator of the start of an outbreak or epidemic (Go *et al.*, 2014).

The mortality rate for NA-EEEV strains, has been reported in horses between 80–90% especially in animals without prior vaccination or with partial immunity, while in humans, the mortality rate varies according to reports between 30% and 70% for neuroinvasive cases of the disease, although the morbidity is between 4–5% (CDC, 2024). In contrast, SA-EEEV strains (MADV), are associated with equine but rarely human disease, though human virulence from SA-EEEV was reported in 2010 in Panama (Rivera *et al.*, 2024). Mortality rate of horses has been reported between 60–80% in Argentina and Brazil during outbreaks.

EEEV infections in birds are usually subclinical; however, disease with high-titred viremia and high mortality rates has been reported in chukar partridges, pheasants, egrets, glossy ibises (*Plegadis falcinellus*), rock doves, house sparrows, psittacine birds, ratites (emus, ostriches), African penguins, chickens (<14 days old), pigeons, Pekin ducks, and whooping cranes. Moreover, occasional cases of encephalitis have also been reported in sheep, cattle, deer, South American camelids (llamas and alpacas), pigs, dogs, goats, bats, and small mammals including rodents (Go *et al.*, 2014).

The WEEV complex contains western equine encephalitis virus (WEEV) and several closely related alphaviruses (Table 1). WEEV is the only virus in this complex of significant medical or veterinary importance in the western hemisphere, though Highlands J virus can also be pathogenic for some species (Go *et al.*, 2014; USDA, 2024). WEEV was first isolated in 1930 from encephalitic horses during

an outbreak in the San Joaquin Valley of California, USA. Between the 1930s and 1950s, Highlands J virus was transmitted from *C. melanura* to songbirds in freshwater swamps (Go *et al.*, 2014).

WEEV is maintained in an enzootic cycle between passerine and wild birds as reservoirs and amplifying hosts, and its specific mosquito vector, *Culex tarsalis*. It has been also suggested that small mammals, like lagomorphs and rodents, can serve as amplification hosts in an epizootic cycle with *Aedes* spp. mosquitoes (Go *et al.*, 2014; USDA, 2024). Humans and equids are also dead-end hosts for WEEV, as they do not develop sufficient viremia to transmit the virus, and horses serve as sentinels' animals during outbreaks.

The mortality rate in horses is between 20–30% while in humans is lower, approximately 3–15%. Morbidity rates for WEEV infections in humans and horses are not available, since many infections remain asymptomatic or undetected.

Clinical cases are seen most often in equids, but they have also been reported in western gray squirrels (*Sciurus griseus*), California ground squirrels (*Citellus beecheyi*) and some birds, including emus, turkeys, pheasants and chukar partridges (USDA, 2024). WEEV has been isolated from a sheep with neurological disease (Vissani *et al.*, 2024) and also from reptiles (e.g. snakes, tortoises) and frogs, proposed as possible overwintering hosts (USDA, 2024).

In North America, last reported clinical cases in horses were in 1998, while the virus was detected for the last time in mosquito pools in 2008. In South America, major confirmed equine outbreaks occurred during the 1970s and 1980s, followed by isolated cases in Argentina (1996), Brazil (2007) and Uruguay (2009). After four decades of intermission period, WEEV re-emerge during the spring of 2023. The outbreak of WEEV occurred in South America in 2023–2024, highlights several important aspects of the epidemiology of these viruses including ecological changes affecting reservoir hosts and mosquito vectors, the role of migratory birds, climatic variations like El Niño-South Oscillants and the immunological status of equine population in the region, which likely contributed to the reported outbreak.

1.2. VEEV complex

The VEE virus complex is composed of six subtypes (I–VI) (Table 1) and can be divided into epizootic (or epidemic) and enzootic (or endemic) viruses, based on their behaviour and amplifying hosts. The epizootic subtypes I-AB and I-C (Subtype I includes five antigenic variants (AB–F), of which variants I-AB and I-C have been associated with major equine epizootics and epidemics involving equids and humans are associated with epizootic VEE in equids and concurrent epidemics in humans (Aguilar *et al.*, 2011; Galisher *et al.*, 1980; Kumar *et al.*, 2018; Monath & Trent, 1981; Pan American Health Organization, 1972; Walton, 1981; Walton *et al.*, 1973; Walton & Grayson, 1989). Originally, subtypes I-A and I-B were considered to be distinct variants, but they are now considered to be identical (I-AB). The epizootic-These variants, I-AB and I-C, are thought to originate from mutations of the enzootic I-D serotype (Weaver *et al.*, 2004); I-AB and I-C isolates have only been obtained during equine epizootics. Epizootic VEEV have been identified in Central and northern South America with the spread into southern USA in 1971 (Kumar, 2018, Weaver *et al.*, 1999a).

Epizootic VEEV strains (I-AB and I-C) are efficiently amplified in an equid–mosquito cycle that typically occurs in agricultural settings. The infection of horses, donkeys and mules with these strains result in high morbidity rates, with high viremia levels that are sufficient for mosquitoes' transmission. Moreover, horses infected with epizootic VEEV strains, can shed virus in body fluids and the virus can spread by direct contact or via aerosols. Several mosquito species have been incriminated as vectors of epizootic VEEV I-AB and I-C, including *Psorophora columbiae*, *Psorophora confinnis*, *Aedes sollicitans*, *Aedes taeniorhynchus*, *Mansonia indubitans* and *Deinocerites pseudus*. During epizootics periods, VEEV can be transmitted from horses to humans (Aguilar *et al.*, 2011; Go *et al.*, 2014; Weaver *et al.*, 2009a).

VEEV strains I-AB and I-C exist in the epizootic cycle for periods of only months to years and are thought to become extinct between outbreaks because the supply of naive horses becomes exhausted through mortality and immunity in survivors. Equine mortality rates have been estimated at 20–80%, while human fatalities occur infrequently, with neurological disease appearing in only 4–14% of cases (Weaver *et al.*, 2004).

There is documented evidence of VEEV I-AB and I-C infections in other species such as sheep, goats, dogs, cats. However, there is no clear evidence indicating that these species play a significant role in the transmission of the disease.

In contrast, the enzootic strains include variants (subtype I varieties I-D, I-E and I-F, and subtypes II, four antigenic variants (A–D) of subtype III, and subtypes IV– to VI). Normally, enzootic VEE viruses do not produce clinical encephalomyelitis in the equine species are typically avirulent for equids (Walton *et al.*,

1973), although they can produce clinical disease in humans that is indistinguishable from that caused by epizootic strains (Aguilar *et al.*, 2011; Weaver *et al.*, 2012). Remarkably, but in 1993 and 1996 in Mexico, the I-E enzootic subtype caused limited epizootics in horses but the strains involved did not amplify efficiently in equids (Estrada-Franco *et al.*, 2004). Enzootic cycles of VEEV subtypes occur from southern USA (Everglade virus) to northern Argentina (Rio Negro virus). These enzootic strains, except for Tonate virus (TONV – VEEV-III B), circulate in specific ecological habitats in transmission cycles between rodents and mosquitoes in the subgenus *Culex (melanoconium)* (Weaver *et al.*, 2012). The principal rodent reservoirs are sylvatic species in the genera *Sigmodon*, *Oryzomys*, *Zigodontomys*, *Heteromys*, *Peromyscus* and *Proechimys*. These animals are frequently found to be infected in nature and develop viremia that is sufficient for vector infection. TONV is maintained in a transmission cycle between birds and the cliff swallow bug, *Oeciacus vicarius* (Aguilar *et al.*, 2011).

Based on results from surveillance studies, a large proportion (0.1–7%) of dengue-like illness in Latin America is caused by VEEV, both epizootic and enzootic strains. Therefore, VEEV represents a serious public health concern (Aguilar *et al.*, 2011).

Many wildlife species are susceptible to enzootic strains of VEEV, and evidence of infection has been found in bats, opossums, gray fox and wild birds. However, there is not sufficient information that these species can act as reservoir hosts.

For up-to-date information on the latest outbreaks and endemic regions, consult WOAAH WAHIS interface¹.

The enzootic variants and subtypes can produce clinical disease in humans (Monath & Trent, 1981; Pan-American Health Organization, 1972; Powers *et al.*, 1997; Walton, 1981; Walton & Grayson, 1989).

Historically, epizootic VEE was limited to northern and western South America (Pan-American Health Organization, 1972). From 1969 to 1972, however, epizootic activity (variant I-AB) occurred in parts of North and Central America. Epizootics of VEE caused by I-AB or I-C virus have not occurred in North America since 1972. Equine and human isolations of epizootic VEE virus were subtype I-C strains from Venezuela in 1993, 1995, 1996, 1999, 2000, 2003 (Navarro *et al.*, 2005) and Colombia in 1995. Additionally, variant I-AB has been isolated from sentinel hamsters in Venezuela (Medina *et al.*, 2015).

The foci of enzootic variants and subtypes are found in areas classified as tropical wet forest, i.e. those areas with a high water table or open swampy areas with meandering sunlit streams. These are the areas of the Americas where rainfall is distributed throughout the year or areas permanently supplied with water. Enzootic viruses cycle among rodents, and perhaps birds, by the feeding of mosquitoes (Monath & Trent, 1981; Pan-American Health Organization, 1972; Walton, 1981; Walton & Grayson, 1989). Enzootic VEE virus strains have been identified in the Florida Everglades (subtype II), Mexico (variant I-E), Central American countries (variant I-E), Panama (variants I-D and I-E), Venezuela (variant I-D), Colombia (variant I-D), Peru (variants I-D, III-C, and III-D), French Guiana (variant III-B and subtype V), Ecuador (variant I-D), Bolivia (variant I-D), Suriname (variant III-A), Trinidad (variant III-A), Brazil (variants I-F, III-A, and subtype IV), and Argentina (subtype VI). In an atypical ecological niche, variant III-B has been isolated in the USA (Colorado and South Dakota) in an unusual association with birds (Aguilar *et al.*, 2009; Monath & Trent, 1981; Pan American Health Organization, 1972; Walton, 1981; Walton & Grayson, 1989), while the Everglades virus is a subtype II VEE virus that infects rodents and dogs in Florida.

Table 1: *Alphaviruses from the EEEV, WEEV and VEEV antigenic complexes, which are distributed in America (adapted from Weaver *et al.*, 1999b)*

Antigenic complex	Species	Antigenic subtype	Antigenic variety	Equine clinical signs	Distribution	Transmission pattern
Eastern equine encephalitis (EEE)	EEEV	Madariaga (MADV)	EEEV-NA EEEV-SA	Encephalitis ^(a) Encephalitis	North America, Caribbean Central and South America	Enzootic
Western equine encephalitis (WEE) ^(b)	WEEV		Several	Encephalitis ^(a)	North, South America	Enzootic

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<u>Antigenic complex</u>	<u>Species</u>	<u>Antigenic subtype</u>	<u>Antigenic variety</u>	<u>Equine clinical signs</u>	<u>Distribution</u>	<u>Transmission pattern</u>
	<u>Highlands</u>			<u>Rare encephalitis</u>	<u>Eastern North America</u>	
	<u>Ft. Morgan</u>			<u>None reported</u>	<u>Western North America</u>	
	<u>Buggy Creek</u>			<u>None reported</u>	<u>Oklahoma (USA)</u>	
	<u>Aura</u>			<u>None reported</u>	<u>South America</u>	
<u>Venezuelan equine encephalitis (VEE)</u>	<u>VEEV</u>	<u>(I) VEE</u>	<u>AB</u>	<u>Encephalitis^(a)</u>	<u>South, Central and North America</u>	<u>Epizootic</u>
			<u>C</u>	<u>Encephalitis^(a)</u>	<u>South, Central America</u>	<u>Epizootic</u>
			<u>D</u>	<u>None or mild febrile illness^(a)</u>	<u>South, Central America</u>	<u>Enzootic</u>
			<u>E</u>	<u>None or mild febrile illness^(a)</u>	<u>Central America</u>	<u>Enzootic</u>
			<u>F</u>	<u>None reported</u>	<u>Brazil</u>	<u>Enzootic</u>
		<u>(II) Everglades</u>		<u>None or mild febrile illness^(a)</u>	<u>Florida (US)</u>	<u>Enzootic</u>
				<u>None or mild febrile illness^(a)</u>	<u>Brazil, Trinidad</u>	<u>Enzootic</u>
		<u>(III) Mucambo virus</u>	<u>A (Mucambo)</u>	<u>None or mild febrile illness^(a)</u>	<u>Brazil, Trinidad</u>	<u>Enzootic</u>
			<u>B (Tonate)</u>	<u>None reported^(a)</u>	<u>French Guiana</u>	<u>Enzootic</u>
			<u>B (Bijou Bridge)^(c)</u>	<u>None reported</u>	<u>western North America</u>	<u>Enzootic</u>
			<u>C (71D-1252)</u>	<u>None reported</u>	<u>Peru</u>	<u>Enzootic</u>
		<u>(IV) Pixuna</u>		<u>None or mild febrile illness^(a)</u>	<u>Brazil</u>	<u>Enzootic</u>
		<u>(V) Cabassou</u>		<u>None reported</u>	<u>French Guiana</u>	<u>Enzootic</u>
		<u>(VI) Rio Negro</u>		<u>None reported^(a)</u>	<u>Argentina</u>	<u>Enzootic</u>

^(a)Encephalitis and febrile illness in humans.

^(b)Fort Morgan virus, Buggy Creek virus, Y62-33 virus and Sindbis virus, are members of the VEEV complex but are not distributed in America and clinical disease in horses has not been reported.

^(c)Bijou Bridge virus is a strain of Tonate virus that was detected in wild birds in the US Rocky Mountains in the 1970s.

2. Biosafety Biosecurity

Epizootic (equine amplification-competent) subtype strains of VEEV (subtypes I-AB and I-C) and NA-EEEV (but not MADV) are Health and Human Services (HHS) Select Agents, which means that they are biological agents that have the potential to pose a severe threat to public health, and animal health

For VEEV, WEEV and EEEV, laboratory manipulations should be carried out at an appropriate biosafety and containment level determined by risk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*). Severe clinical disease and death caused by EEE VEEV and WEEV viruses have been documented after laboratory-associated infections in laboratory workers (American Committee on Arthropod-Borne Viruses [ACAV], 1980). The primary laboratory hazards are parenteral inoculation, contact of the virus with broken skin or mucous membranes, bites from infected animals or arthropods, or aerosol inhalation, the viruses are highly infectious by the aerosol route (CDC, 2020). It is The Centers for Disease Control and Prevention National Institutes of Health recommended that personnel be immunised against EEE virus (United States Department of Health and Human Services, 2009) diagnostic and research activities involving clinical material, infectious cultures, and infected animals or arthropods should be performed in appropriate biosafety and containment levels determined by risk analysis, with the corresponding containment equipment, and facilities, included an HEPA filtration as a requirement on the exhaust system of laboratory and animal facilities when using VEEV (CDC, 2020). Precautions should also be taken to prevent human infection when performing post-mortem examinations on horses suspected of being infected with the equine encephalomyelitis viruses. Human VEEV infections have originated by aerosol transmission from the cage debris of infected laboratory rodents

and from laboratory accidents (Quiroz *et al.*, 2009). Infections with both epizootic and enzootic variants and subtypes have been acquired by laboratory workers (ACAV, 1980).

It is recommended that personnel be immunised against VEEV and EEEV (CDC, 2020). Severe clinical disease or death can occur in humans. Those who handle infectious VEEV viruses or their antigens prepared from infected tissues or cell cultures should be vaccinated and shown to have VEEV-specific neutralising antibody (Berge *et al.*, 1961; Pan American Health Organization, 1972). If vaccination is not a viable option, additional personal protective equipment to include respiratory protection is recommended for all procedures. Laboratory manipulations should be carried out at an appropriate biosafety and containment level determined by risk analysis (see chapter 1.1.4).

B. DIAGNOSTIC TECHNIQUES

Confirmatory diagnosis of EEEV, WEEV or VEEV is by virus isolation or virus identification by real-time reverse-transcription polymerase chain reaction (RT-PCR) and further sequencing. Several neurological conditions should be considered in the differential diagnosis, including other viral encephalomyelitides (rabies, EHV-1 myeloencephalopathy, flavivirus encephalitis, Borna disease virus), bacterial meningitis, leukoencephalomalacia, lead poisoning, equine protozoal myeloencephalitis, nigropallidal encephalomalacia, botulism, and verminous encephalitis.

1. Detection of the agent

1.1. Collection and preparation of specimens

1.1.1. Tissue samples

Virus isolation and molecular detection can be attempted from central nervous system (CNS) tissue (brain, cerebellum and, spinal cord) and other tissues (such as liver and spleen) of horses with neurological disease suspected to be infected with equine alphaviruses, unless more than 5 days have elapsed between the appearance of clinical signs and the death of the horse. Tissue samples should be transported to the laboratory refrigerated within 48 hours of collection; otherwise, they should be frozen and sent with dry ice. At the laboratory, samples should be held at 4°C until preparation. Samples that cannot be processed within a few hours should be stored at -70°C. Samples should be handled in biosafety cabinet (class II) at the appropriate biosafety laboratory level determined by risk analysis (described previously in Section A.2). The supernatant obtained is collected and can be used for viral isolation (described below in Section B.1.2) or further reverse-transcription polymerase chain reaction (RT-PCR) detection (described below in Section B.1.3) following RNA extraction using an appropriate kit or robotic system.

1.1.2. Blood

Molecular detection by RT-PCR, particularly of epidemic strains of VEEV, can be attempted from the blood of live animals during the early febrile stage of the illness; however, viraemia usually disappears by the time neurological signs appear (USDA, 2024). A sample of 10–20 ml whole blood should be collected using an aseptic technique. RNA is extracted using an appropriate kit or robotic system, and RT-PCR detection is undertaken (described below in Section B.1.3).

Virus detection in apparently healthy animals is very low-yield and inefficient, and therefore not cost-effective, and should not be considered for routine surveillance programmes (USDA, 2024).

Table 1-2. Test methods available for the diagnosis of EEEV and WEEV and VEE and their purpose²

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases ^(a)	Prevalence of infection – surveillance ^(a,b)	Immune status in individual animals or populations post-vaccination ^(b)
Detection of the agent ^(c)						
RT-PCR	□□□	☑☑☑ □□□	□□□	☑☑☑	□□□	□□□

2 — For WEE and VEE, not all test modalities have been thoroughly evaluated in naturally infected horses

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases ^(a)	Prevalence of infection – surveillance ^(a, b)	Immune status in individual animals or populations post-vaccination ^(b)
<u>Virus Isolation</u> Isolation in cell culture	□□□	☑☑☑ □□□	□□□	☑☑☑	□□□	□□□
<u>In-situ hybridisation</u>	□□□	□□□	□□□	☑□□	□□□	□□□
Detection of immune response						
IgM capture-ELISA	□□□	☑□□	□□□	☑☑□	□□□	□□□
Plaque reduction neutralisation ^(d)	☑☑☑	☑□□ □□□	□□□	☑☑□	☑☑☑	☑☑☑
<u>Virus neutralisation</u>	☑☑☑	□□□	□□□	□□□-☑☑□	☑☑☑	☑☑☑
Haemagglutination-inhibition (paired samples)	☑□□	☑☑□	□□□	☑☑□	☑☑□	☑☑□
Complement fixation (paired samples)	□□□	☑□□	□□□	☑☑□	□□□	□□□

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;

IgM ELISA = immunoglobulin M-enzyme-linked immunosorbent assay.

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

^(d)Surrogate virus neutralisation tests can be performed under reduced biosafety conditions, as determined by biological risk assessment.

Table 3. Test methods available for the diagnosis of VEEV and their purpose

Method	Purpose					
	<u>Population freedom from infection</u>	<u>Individual animal freedom from infection prior to movement</u>	<u>Contribute to eradication policies</u>	<u>Confirmation of clinical cases^(a)</u>	<u>Prevalence of infection – surveillance^(a, b)</u>	<u>Immune status in individual animals or populations post-vaccination^(b)</u>
<u>Detection of the agent^(c)</u>						
<u>RT-PCR</u>	□□□	☑☑☑	□□□	☑☑☑	□□□	□□□
<u>Virus isolation</u>	□□□	□□□	□□□	☑☑☑	□□□	□□□
<u>Whole genome sequencing</u>	□□□	□□□	□□□	☑☑☑	□□□	□□□
<u>Detection of immune response</u>						
<u>Plaque reduction neutralisation</u>	☑☑☑	□□□	□□□	☑☑□	☑☑☑	☑☑☑
<u>Virus neutralisation</u>	☑☑☑	□□□	□□□	☑☑□	☑☑☑	☑☑☑

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.

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

1.1.2. In-vitro and in-vivo culture Virus isolation

The definitive method for diagnosis of EEE or WEE is virus isolation followed by typing. EEE virus can usually be isolated from the brains of horses, unless more than 5 days have elapsed between the appearance of clinical signs and the death of the horse. EEE virus can frequently be isolated from brain tissue even in the presence of a high serum antibody titre. WEE virus is rarely isolated from tissues of infected horses. Brain is the tissue of choice for virus isolation, but the virus has been isolated from other tissues, such as the liver and spleen.

During VEE infection, viraemia coincides with the onset of pyrexia within 12–24 hours of infection. Viraemia usually terminates 5–6 days after the onset of infection, and coincides with the production of neutralising antibodies and the appearance of clinical neurological signs. Frequently, VEE viruses cannot be isolated from the brains of infected equids. Blood samples for virus isolation should be collected from febrile animals that are closely associated with clinical encephalitic cases. It is recommended that a set of these tissues be collected in duplicate, one set for virus isolation and the other set in formalin for histopathological examination. Specimens for virus isolation should be sent refrigerated if they can be received in the laboratory within 48 hours of collection; otherwise, they should be frozen and sent with dry ice. A complete set of tissues will allow the performance of diagnostic techniques for other diseases. For isolation, a 10% suspension of tissue is prepared in phosphate buffered saline (PBS), pH 7.8, containing bovine serum albumin (BSA) (fraction V; 0.75%), penicillin (100 units/ml), and streptomycin (100 µg/ml). The suspension is clarified by centrifugation at 1500 *g* for 30 minutes.

1.2.1. Virus isolation in cell cultures

EEEV, WEEV and VEEV viruses can be isolated in a number of cell culture systems. The most commonly used cell cultures are mammalian primary chicken or duck embryo fibroblasts, continuous cell lines of African green monkey kidney (Vero), rabbit kidney (RK-13), or baby hamster kidney (BHK-21) and mosquito cell lines (e.g. C6/36) (CDC, 2024; USDA, 2024). Isolation is usually attempted in 25 cm² cell culture flasks. Confluent cells are inoculated with 1.0 ml of tissue suspension homogenate. Following a 1–2-hour absorption period, cell monolayer is rinsed twice with culture medium or phosphate buffered saline (PBS), and maintenance medium is added. Cultures are incubated for 6–8 days, and One blind passage is made can be performed if cytopathic effects are not visualised in the first passage (Logue *et al.*, 2009; Vissani *et al.*, 2024). EEEV, WEEV and VEEV viruses will produce a cytopathic change in cell culture. Cultures that appear to be infected are frozen. The fluid from the thawed cultures is used for virus identification by real-time RT-PCR and further sequencing (described below in Sections B.1.3 and B.1.4).

Tissue suspensions can be inoculated by the yolk sac route into 6 to 8 day old embryonating chicken eggs. There are no diagnostic signs or lesions in the embryos infected with these viruses. Inoculated embryos should be incubated for 7 days, but deaths usually occur between 2 and 4 days post-inoculation. Usually only one passage is made unless there are dead embryos, from which virus cannot be isolated.

Viral isolates can be identified by reverse transcription polymerase chain reaction (RT-PCR), by direct or indirect fluorescent antibody test, or by plaque reduction neutralisation (PRN) tests using polyclonal or monoclonal antibodies to specific viruses obtained commercially or prepared via hyperimmunisation of animals with further collection of their serum (or ascites fluid in case of mice) and purification of immunoglobulins.

1.2.2. Virus isolation in mice

For those cases in which virus isolation in cell culture cannot be achieved and cytopathic effects are not visualised during passages, inoculation in mice can be performed as an alternative method. The mice model is a well-established method; however, the use of this technique for diagnosis should be minimised to reduce the use of laboratory animals (Beatty *et al.*, 1995; Linssen *et al.*, 2000; Del Piero *et al.*, 2001).

Animals should be maintained in a facility under biosafety conditions, as determined by biological risk assessment, and housed in microisolator cages. Specific pathogen-free suckling mice are inoculated subcutaneously with an inoculum of 0.1–0.2 ml of the clarified supernatant of a 10% (w/v) homogenate of tissue (described previously in Section B.1.1). The mice are observed daily for 14 days. Organs of dead mice are dissected, weighed, and homogenised in PBS/medium to give a 20% (w/v) suspension. Homogenates are clarified and examined for equine encephalitis

by RT-PCR. Any deaths occurring during the first 24 hours are regarded as nonspecific (due to stress/bacterial infection, etc.) (Paessler *et al.*, 2004).

1.2.3. Molecular methods – detection of nucleic acid

Diagnosis by RT-PCR is rapid, sensitive, and does not depend on the presence of infectious virus in the clinical sample. PCR methods are particularly useful in cases of neurological disease, in which rapid identification is critical for guiding management strategies and communication to health authorities. Real-time (or quantitative) RT-PCR has become the method of choice for the majority of diagnostic laboratories and provides rapid and sensitive detection of viral RNA. Moreover, it can be performed under reduced biosafety conditions and is also available in high throughput formats.

1.2.3.1. Conventional reverse-transcription PCR

Several methods of RT-PCR for detection of EEE, WEE or VEE viral RNA in mosquitoes and vertebrate tissues have been described, although few have been extensively validated for mammalian samples (Linssen *et al.*, 2000; Monroy *et al.*, 1996; Vodkin *et al.*, 1993).

Table 4 summarises the primer sequences for some of the most widely used assays, while the optimised thermocycler times and temperatures are documented in the publications cited.

An alpha-universal nested RT-PCR (RT-nPCR) targeting the nsP4 region was described by Sanchez-Seco *et al.* (2001) based on the nucleotide similarity among alphaviruses in the nsP4. Reverse transcription of RNA to cDNA should be carried out first. This assay was designed for rapid detection and identification of alphaviruses, and consists of an RT-PCR followed by a nested PCR and sequencing. Primer sequences used for detection of alphavirus viral RNA are shown in Table 4. Primers 1+ and 1-, and 2+ and 2-, are used for the first and second PCR rounds, respectively. This RT-nPCR is a specific and sensitive tool for alphavirus detection and identification, allowing rapid and reliable detection of every distinct virus belonging to the alphavirus genus (EEEV, VEEV, WEEV, Sindbis virus, Chikungunya virus, Semliki Forest virus and Ross River virus). This methodology does not amplify nonrelated RNA, such as from Vero cell cultures, flaviviruses or rubiviruses.

A multiplex ~~nested~~ RT-nPCR method was developed to expedite differential diagnosis in cases of suspected EEEV or ~~West Nile arboviral~~ WNV encephalomyelitis in horses (Johnson *et al.*, 2003). It has been shown to be sensitive and specific for NA-EEEV and WNV, providing a rapid means of identifying both viruses in brain tissues. The assay has enhanced speed and sensitivity compared with cell culture virus isolation and has been used extensively in the US-NVSL³ during several subsequent arbovirus seasons. Primer sequences used at NVSL for detection of EEEV, WEEV and VEEV viral RNA are shown in Table 2.4. The first reaction is performed ~~in is one tube~~ and starts with the reverse transcription at 46–50°C (depending on the optimum temperature range of the enzyme used) for 30 minutes followed by polymerase activation at 95°C for 15 minutes. Then, 35 cycles consisting of denaturation at 94°C for 45 seconds; annealing at 58°C for 45 seconds, and elongation at 72°C for 1 minute are performed. An additional elongation step at 72°C for 9 minutes is applied at the end of the reaction. The nested PCR uses 1 µl of the first PCR product in 50 µl of reaction mix, and is performed under the following cycling conditions: polymerase activation at 95°C for 15 minutes, 35 cycles consisting of the steps described above with annealing temperature 46°C, followed by the final elongation at 72°C for 9 minutes.

An approach aimed at detecting variants of VEEV ~~virus~~ using one pair of degenerated primers (Table 2.4) was also developed (Pisano *et al.*, 2012). For this method, the nsP1 region was selected because of the high homology shown between all VEEV subtypes and the difference shown with the rest of the alphaviruses. This RT-nPCR amplifies a short and conserved fragment throughout VEE complex members for detection of all VEE viruses. The sensitivity has proven to be slightly lower than viral isolation, being able to detect <500 plaque-forming units (PFU). Following reverse transcription at 46–50°C (depending on the enzyme used) for 30 minutes and denaturation at 94°C for 2 minutes, the authors recommend first PCR (40 cycles) consisting of denaturation at 94°C for 30 seconds; annealing at 64°C for 60 seconds; and elongation at 72°C for 30 seconds, supplemented with a final extension at 72°C for 5 minutes. For the nested reaction, 2 µl of the first PCR product is mixed in 50 µl of reaction mix and subjected to initial denaturation at 94°C for 2 minutes, followed by 40 cycles of amplification consisting of denaturation at 94°C for 30 seconds; annealing at 61°C for 40 seconds; and elongation at 72°C for 30 seconds. A final extension at 72°C for 5 minutes is then applied.

3 NVSL: National Veterinary Service Laboratories (of the USA).

Table 2 4. Primers used for detection of *EEE*, *WEE* and *VEE* viral RNA via nested RT-PCR

Virus	Genome position	Amplicon length	Primer sequence (5'–3')	Source
pan-Alphavirus (1 st stage)	6137–6485	481 bp	F: GAY-GCI-TAY-YTI-GAY-ATG-GTI-GAIG-G R: KYT-CYT-CIG-TRT-GYT-TIG-TIC-CIG-G	Sanchez-Seco <i>et al.</i> , 2001
pan-Alphavirus (nested)	6315–6593	278 bp	F: GIA-AYT-GYA-AYG-TIA-CIC-ARA-TG R: GCR-AAI-ARI-GCI-GCI-GCY-TYI-GGI-CC	Sanchez-Seco <i>et al.</i> , 2001
EEE (1 st stage)	9233–9797	565	F: AGG-GCT-TAC-CTG-ATT-GAC R: GTA-ACG-CCA-GGA-GTA-TTG	NVSL
EEE (nested)	9571–9710	140	F: GGC-TCA-AGA-GTC-AGG-AGA R: CGG-ATG-TGA-CAC-AAG-AGA	NVSL
WEE (1 st stage)	9032–9621	590	F: TAA-GTG-TGG-CGA-CTA-CAG R: TCA-GGC-AGT-CTC-TTC-TTG	NVSL
WEE (nested)	9241–9575	335	F: CTC-ACA-CGC-CTA-CAG-TCA R: AGT-GCC-TAC-CAG-GAT-AGC	NVSL
VEE I-AB/C/D (1 st stage)	9215–9776	562	F: AGC-CAG-TGC-ACA-AAG-AAG R: TAG-GTG-TTA-GCC-GGT-AAG	NVSL
VEE I-AB/C/D (nested)	9536–9671	136	F: GGG-TGG-GAG-TTT-GTA-TGG R: CCA-GGA-TGG-TGG-ACA-TAG	NVSL
VEE I-E (1 st stage)	9611–10085	475	F: GTA-ATC-CAC-ACG-GAC-TAC R: GCA-TAA-CCC-GCT-CTG-TTG	NVSL
VEE I-E (nested)	9794–9955	162	F: GCA-TGC-CTC-TGT-GCT-TAG R: ATT-TCA-GCA-AGC-GGG-TAG	NVSL
VEE all (1 st stage)	45–176 <u>201</u>	156	F: ATG-GAG-AAR-GTT-CAC-GTT-GAY-ATC-G R: YTC-GAT-YAR-YTT-NGA-NGC-YAR-ATG-C	Pisano <i>et al.</i> , 2012
VEE all (nested)	83–163	80	F: ARG-AYA-GYC-CNT-TCC-TYM-GAG-C R: CRT-TAG-CAT-GGT-CRT-TRT-CNG-TNA-C	Pisano <i>et al.</i> , 2012

F: forward; R: reverse.

A combination of an RT-PCR with an enzyme-linked immunosorbent assay (ELISA: RT-PCR-ELISA) was reported as a method to identify alphaviruses that are pathogenic to humans (Wang *et al.*, 2006).

1.3.2. Real-time reverse-transcription PCR

Currently real-time RT-PCR is the preferred method of detection of alphaviruses (described previously in Section B.1.3). A multiplexed detection of *EEEV* and *WEEV* viral RNA via real-time RT-PCR has been reported (Kang *et al.*, 2010), but the method has not been extensively evaluated in field samples. A sensitive real-time RT-PCR for detection of North American variants of *EEEV* and *WEEV* viral RNA has been developed and validated on a limited panel of field samples; it demonstrated a superior sensitivity compared with virus isolation in Vero cell culture (Lambert *et al.*, 2003). The primers and probes (Table 3 4) were evaluated with a number of synthetic RNA constructs that incorporated various substitutions present in North American *EEEV* and *WEEV* viral variants (Vina-Rodriguez *et al.*, 2016). This confirmed the high sensitivity and specificity of the reagents developed by Lambert *et al.* (2003). This assay was extensively used for the detection of *WEEV* during the outbreak in South America (Campos *et al.*, 2024; Vissani *et al.*, 2024). The real-time RT-PCR starts with reverse transcription at 46–50°C (depending on the enzyme used) for 30 minutes followed by *Taq* polymerase activation at 95°C for 15 minutes. Then, 45 cycles consisting of denaturation at 95°C for 15 seconds; annealing at 55°C for 30 seconds; and elongation at 72°C for 30 seconds are performed.

Real-time RT-PCR for detection of *VEEV* virus (Vina-Rodriguez *et al.*, 2016) was evaluated on a set of synthetic RNA oligonucleotides and needs further validation with field samples of *VEE* virus *VEEV*. This is particularly important because the nsP1 genome area where the primers and probes were selected is highly conserved in alphaviruses (Eshoo *et al.*, 2007), and the possibility of undesired cross-reactivity needs to be ruled out.

Table 3 5. Real-time RT-PCR primers and probes to *EEE*, *WEE* and *VEE* viruses. Both probes are labelled with FAM and quenched with BHQ1 (Lambert *et al.*, 2003)

Virus	Reagent designation	Genome position	Sequence (5'–3')	Amplicon length
EEE	EEE 9391, primer F	9391–9411	ACA-CCG-CAC-CCT-GAT-TTT-ACA	69
	EEE 9459c, primer R	9459–9439	CTT-CCA-AGT-GAC-CTG-GTC-GTC	
	EEE 9414 Probe	9414–9434	TGC-ACC-CGG-ACC-ATC-CGA-CCT	
WEE	WEE 10,248 primer F	10,248–10,267	CTG-AAA-GTC-GGC-CTG-CGT-AT	67
	WEE 10,314c primer R	10,314–10,295	CGC-CAT-TGA-CGA-ACG-TAT-CC	
	WEE 10,271 Probe	10,271–10,293	ATA-CGG-CAA-TAC-CAC-CGC-GCA-CC	
VEE	AlphaVIR966 primer F	151–178	TCC-ATG-CTA-ATG-CYA-GAG-CGT-TTT-CGC-A	98
	AlphaVIR966 primer R	248–225	TGG-CGC-ACT-TCC-AAT-GTC-HAG-GAT	
	INEID-VEEV Probe	193–218	TGA-TCG-ARA-CGG-AGG-TRG-AMC-CAT-CC	

F: forward; R: reverse.

1.3.3. Whole genome sequencing

Although real time RT-PCR is the method of choice for diagnosis of alphaviruses, whole genome sequencing is the selected method for its characterisation, principally to define the VEEV subtypes/variants involved after a positive RT-PCR result, which is critically important to understand why certain strains are likely to cause epizootic infection, and to forecast the incidence of potential epidemic events.

As the VEEV genome is small, whole genome multiple sequence alignment (MSA) of more than 140 sequences is feasible. However, a high-resolution genome-wide single nucleotide polymorphism microarray assay was described (Gardner *et al.*, 2016). This microarray successfully genotyped VEEV directly from tissue samples of infected mice, bypassing the need for viral isolation, culture and genomic sequencing of single genes. This tool allowed to determine genomic variants associated with serotypes and host species, revealing a complex relationship between genotype and phenotype, being particularly useful for rapidly evaluating a novel strain from an epizootic outbreak event.

1.3.4. *In-situ* hybridisation

In-situ hybridisation (ISH) is a molecular biology technique used to detect the presence, determine viral tropism/distribution and viral load of specific RNA sequences within formalin-fixed, paraffin-embedded tissues. While viral isolation and RT-PCR are considered the gold standards for the confirmation of alphaviruses infection in clinical cases, ISH is especially useful in cases where fresh tissues are not available or not suitable for virus isolation/RT-PCR.

In the context of EEEV and WEEV, ISH can be used either to detect or to differentiate both viruses within formalin-fixed, paraffin-embedded tissues of horses with neurological problems that have died. ISH procedures have been described for EEEV (Pennick *et al.*, 2012) and for WEEV (Vissani *et al.*, 2024). Both ISH described are highly specific and sensitive; however, failure to identify viral antigen in equine CNS by ISH does not rule out infection. No ISH procedures are available for detection and characterisation of VEEV.

1.34. Antigen detection

Antigen capture ELISA has been developed for EEE surveillance in mosquitoes. This can be used in countries that do not have facilities for virus isolation or RT-PCR (Brown *et al.*, 2001).

Although there are a few reports on the use of immunohistochemical (IHC) procedures carried out on fixed tissues using an antibody detection system (Del Piero *et al.*, 2001; Pennick *et al.*, 2012), ~~are~~ IHC is ~~very~~ useful for diagnosis of EEE when fresh tissues are not available or not suitable for virus isolation/RT-PCR, as they are carried out on fixed tissues (Pennick *et al.*, 2012). The envelope protein of EEEV virus is targeted in IHC. ~~Necrotic and inflamed areas of the brain are examined.~~ In EEEV virus infected horses, positive staining is observed most notably in neurons and associated dendritic processes. ~~However, Failure to identify viral antigen in equine central nervous system does not rule out infection. No IHC procedures are available for identification of WEEV and VEEV.~~

2. Serological tests

Serological confirmation of EEE or WEE virus infection requires a four-fold or greater increase or decrease in antibody titre in paired serum samples collected 10–14 days apart.

Arboviral diagnostic serology is complex because of the wide variety of viruses. Due to its specificity and sensitivity, the gold standard tests to detect antibodies against equine alphaviruses remain the virus-neutralisation test (VNT) and the plaque reduction neutralisation test (PRNT). Although the IgM antibody-capture enzyme-linked immunosorbent assay (ELISA) is a valuable tool for rapid diagnosis of acute viral infections in humans, reagents for equine diagnosis are not commercially available worldwide and no standardised and validated methods for WEEV, EEEV and VEEV have been published (only *in-house* tests) (CDC, 2024). In the same way, there are no standardised IgG ELISA protocols for equine alphaviruses. Complement fixation and hemagglutination inhibition tests have also been widely used in the past; however, these tests are technically demanding making reproducibility difficult, and cross reactions may occur between EEEV and WEEV.

Antibodies can cross-react between different equine encephalitis viruses, meaning that antibodies produced against one virus can also bind to and neutralise other related viruses. Studies have shown that antibodies against EEEV or WEEV can provide some level of protection against VEEV. Most horses infected with EEE or WEE virus have a high antibody titre when clinical disease is observed. Consequently, a presumptive diagnosis can be made if an unvaccinated horse with appropriate clinical signs has antibody against only EEE or WEE virus. In contrast to EEE and WEE virus infection, onset of clinical signs and subsequent IgG antibody production against VEE virus occurs early post infection. Development of encephalitis is variable, thus some neurologic horses may not exhibit a four-fold or greater increase in antibody titre in paired sera. The detection of IgM antibody by the ELISA can be suggestive for an acute infection or a recent exposure to the virus, including recent vaccination, therefore interpretation of serological findings must be done in conjunction with clinical signs and the epizootic situation (Sahu *et al.*, 1994).

Vaccination history must be taken into account when interpreting results of any serological tests, particularly the PRN or virus neutralisation (VN) tests. Although enzootic VEE subtypes and variants are non-pathogenic for equids, infection will stimulate antibody production that can cross-react on diagnostic tests with epizootic VEE virus variants. Furthermore, there may be cross-reactions between antibody against EEE and WEE virus in such tests as complement fixation (CF) and haemagglutination inhibition (HI) tests. CF antibodies against both EEE and WEE viruses appear later and do not persist; consequently, the CF test is less useful for the serological diagnosis of disease.

Serum should be collected as soon as possible after the onset of clinical signs (acute phase) and repeated 2 weeks later (convalescent phase) for comparison. Cerebrospinal fluid from horses with neurological problems can also be analysed by serology.

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 phases of the disease. However, due to the high lethality of EEEV and VEEV, and in hyperacute cases of WEEV, it might not be possible to conduct paired serum sampling due to the short clinical duration of the disease. In this case, clinical signs, pathological signs, and epidemiological links would provide further evidence to substantiate a case.

Live virus is used in the PRNT or VNT procedures, which currently requires appropriate biosafety and containment levels determined by risk analysis. These requirements restrict the ability of public health laboratories to conduct PRNT and VNT. Sindbis virus (SINV)/EEEV recombinant constructs have been engineered to express the immunogenic structural proteins from two wild-type EEEV strains in an attenuated form. These SINV/EEEVs, which are not classified as select agents, have been evaluated as alternative diagnostic reagents in a PRNT using human, equine, and murine sera showing specificity comparable to that of wild-type EEEV, with only a slight reduction in sensitivity (Johnson *et al.*, 2011). Also, recombinant SINV/VEEV constructs engineered to express structural proteins from multiple VEEV subtypes were evaluated as diagnostic reagents in VEEV-specific serological assays showing to be effective as the parental virus for identifying infection of humans, horses, and rodents in these serological assays (Ni *et al.*, 2007). These surrogate viruses with equivalent antigenic makeup that confer equivalent sensitivity and specificity to the wild-type viruses should be highly useful in diagnostic laboratories throughout the Americas.

2.1. Complement fixation

The CF test is frequently used for the demonstration of antibodies, although the antibodies detected by the CF test may not persist for as long as those detected by the HI or PRN tests. A sucrose/acetone mouse brain extract is commonly used as antigen. The positive antigen is inactivated by treatment with 0.1% beta-propiolactone. The effectiveness of this treatment should be confirmed by viability testing using *in-vivo* or *in-vitro* culture (see Section B.1.1) prior to working with the antigen at the bench.

In the absence of an international standard serum, the antigen should be titrated against a locally prepared positive control serum. The normal antigen, or control antigen, is mouse brain from uninoculated mice similarly extracted and diluted.

Sera are diluted 1/4 in veronal buffered saline containing 1% gelatin (VBSG), and inactivated at 56°C for 30 minutes. Titrations of positive sera may be performed using additional twofold dilutions. The CF antigens and control antigen (normal mouse brain) are diluted in VBSG to their optimal amount of fixation as determined by titration against the positive sera; guinea-pig complement is diluted in VBSG to contain 5 complement haemolytic units/50% (CH₅₀). Sera, antigen, and complement are reacted in 96-well round-bottom microtitre plates at 4°C for 18 hours. The sheep red blood cells (SRBCs) are standardised to 2.8% concentration. Haemolysin is titrated to determine the optimal dilution for the lot of complement used. Haemolysin is used to sensitise 2.8% SRBCs and the sensitised cells are added to all wells on the microtitre plate. The test is incubated for 30 minutes at 37°C. The plates are then centrifuged (200 g), and the wells are scored for the presence of haemolysis. The following controls are used: (a) serum and control serum each with 5 CH₅₀ and 2.5 CH₅₀ of complement; (b) CF antigen and control antigen each with 5 CH₅₀ and 2.5 CH₅₀ of complement; (c) complement dilutions of 5 CH₅₀, 2.5 CH₅₀, and 1.25 CH₅₀; and (d) cell control wells with only SRBCs and VBSG diluent. These controls test for anticomplementary serum, anticomplementary antigen, activity of complement used in the test, and integrity of the SRBC indicator system in the absence of complement, respectively.

To avoid anticomplementary effects, sera should be separated from the blood as soon as possible after collection and appropriate clotting has occurred. Positive and negative control sera should be used in the test.

2.2. Haemagglutination inhibition

The antigen for the HI test is the same as described above for the CF test. The antigen is diluted so that the amount used in each haemagglutinating unit (HAU) is from four to eight times that which agglutinates 50% of the RBCs in the test system. The haemagglutination titre and optimum pH for each antigen are determined with goose RBCs diluted in pH solutions ranging from pH 5.8 to pH 6.6, at 0.2 intervals.

Sera are diluted 1:10 in borate saline, pH 9.0, and then inactivated at 56°C for 30 minutes. Kaolin treatment is used to remove nonspecific serum inhibitors. Alternatively, nonspecific inhibitors may be removed by acetone treatment of serum diluted 1:10 in PBS followed by reconstitution in borate saline. Sera should be absorbed before use by incubation with a 0.05 ml volume of washed packed goose RBCs for 20 minutes at 4°C.

Following heat inactivation, kaolin treatment and absorption, twofold dilutions of the treated serum are prepared in borate saline, pH 9.0 with 0.4% bovalbumin. Serum dilutions (0.025 ml/well) are prepared in a 96-well round bottom microtitre plate in twofold dilutions in borate saline, pH 9.0, with 0.4% bovalbumin. Antigen (0.025 ml/well) is added to the serum. Plates are incubated at 4°C overnight. RBCs are derived from normal white male geese⁴ and washed three times in dextrose/gelatin/veronal (DGV), and a 7.0% suspension is prepared in DGV. The 7.0% suspension is then diluted 1:24 in the appropriate pH solution, and 0.05 ml per well is added immediately to the plates. Plates are incubated for 30 minutes at 37°C. Positive and negative control sera are incorporated into each test. A test is considered to be valid only if the control sera give the expected results. Titres of 1:10 and 1:20 are suspect, and titres of 1:40 and above are positive.

2.3. Enzyme-linked immunosorbent assay

Several kits for IgM detection from equine specimens are commercially available. The ELISA is performed by coating flat bottomed plates with anti-equine IgM capture antibody (Sahu *et al.*, 1994). The example below provides a generic description of the procedure, which may vary depending on the recommendations developed by the manufacturer to achieve the optimum balance between sensitivity and specificity of the test.

The anti-equine IgM antibody is diluted in 0.5 M carbonate buffer, pH 9.6, and 50–100 µl of this solution is added to each well of a 96-well plate. The plates are incubated at 37°C for 1 hour, and then at 4°C overnight. Prior to use, the coated plates are washed three times with 200–300 µl/well of 0.01 M PBS containing 0.05% Tween 20. After the second wash, 200 µl/well of PBS/Tween/5% non-fat dried milk (or another blocking reagent) is added and the plates are incubated at room temperature for 1 hour. Following incubation, the plates are washed again three times with PBS/Tween. Test and control sera

⁴—RBCs from adult domestic white male geese are preferred, but RBCs from other male geese can be used. If cells from female geese are used, there may be more test variability. It has been reported that rooster RBCs cause a decrease in the sensitivity of the test.

are diluted 1/400 in 0.01 M PBS, pH 7.2, containing 0.05% Tween 20, and 50 µl is added to each well. The plates are incubated at 37°C for 90 minutes and then washed three times. Next, 50 µl of viral antigen is added to all wells. The plates are incubated overnight at 4°C, and washed three times. Then, 50 µl of horseradish peroxidase-conjugated monoclonal antibody (MAb) specific to the viral antigen used is added. The plates are incubated for 60–90 minutes at 37°C and then washed six times. Finally, 50 µl of freshly prepared ABTS (2,2' azino-bis [3-ethylbenzo-thiazoline-6-sulphonic acid]) substrate and hydrogen peroxide (0.1%) is added, and the plates are incubated at room temperature for 15–40 minutes. The light absorbance is measured at 405 nm. A test sample is considered to be positive if the absorbance of the test sample in wells containing virus antigen is at least twice the absorbance of negative control serum in wells containing virus antigen and at least twice the absorbance of the sample tested in parallel in wells containing negative control antigen. To ensure specificity, each serum sample is tested for reactivity with both virus antigen and control antigen.

2.41. Plaque reduction neutralisation

The PRNT test is very specific and can be used to differentiate between EEEV, WEEV and VEEV virus infections. The PRNT test is performed in duck embryo fibroblast, Vero, or BHK-21 cell cultures in 25 cm² flasks, or six 12-well plates, and six-well plates and Microneutralisation 96-well microtiter plates. Volumes listed below are for the flasks, and should be halved if test is performed in six-well plates.

2.1.1. Test procedure

- i) Prior to testing, serum is. Sera are heat-inactivated at 56°C for 30 minutes.
- ii) It can be screened at a 1/10 and 1/100 final dilution. Endpoints can be established using the PRNT or HI tests if serial serum dilutions (e.g. 2-fold, 5-fold, 10-fold, etc.) are tested. This is particularly useful for paired samples obtained from one animal and separated by several days or weeks.
- iii) Serum used in the PRNT test is tested against 100 plaque-forming units (PFU) of virus (50 PFU for six-well plates).
- iv) The virus/serum mixture is incubated at 37°C for 75 minutes before inoculation onto confluent cell culture monolayers in 25 cm² flasks.
- v) The inoculum is adsorbed for 1 hour, followed by the addition of 6 ml of overlay medium. The overlay medium consists of two solutions that are prepared separately. Solution I contains 2 × Earle's Basic Salts Solution without phenol red, 4% fetal bovine serum (FCS), 100 µg/ml gentamicin, 200 µg/ml nystatin, 0.45% solution of sodium bicarbonate, and 0.002% neutral red. When duck embryo fibroblasts are used, Solution 1 also contains 6.6% yeast extract lactalbumin hydrolysate. Solution II consists of 2% Noble agar that is sterilised and maintained at 47°C. Equal volumes of solutions I and II are adjusted to 47°C and mixed together just before use.
- vi) The test is incubated for 48–72 hours, and endpoints are based on a 90% reduction in the number of plaques compared with the virus control flasks, which should have about 100 plaques.

2.2. Virus neutralisation

This test is most commonly performed in flat-bottomed 96-well microtitre plates (tissue culture grade) using a constant dose of virus and doubling dilutions of equine test sera. At least three replicate wells for each serum dilution are required. Maintenance medium (Dulbecco's Modified Eagle's Medium: DMEM) with a concentration of 10% FCS is used throughout as a diluent. Virus stocks of known titre are diluted just before use to contain 100 TCID₅₀ (50% tissue culture infective dose) in 50 µl. Monolayers of Vero cells are prepared. Antibody positive and negative control equine sera and controls for cell viability, virus infectivity, and test serum cytotoxicity, must be included in each assay. End-point VN titres of antibody are calculated by determining the reciprocal of the highest serum dilution that protects ≥75% of the cell monolayer from virus destruction in the replicate wells.

2.2.1. Test procedure

- i) Inactivate serums samples and controls at 56°C (± 1°C) for 30 minutes.
- ii) In 96-well flat-bottomed sterile culture plates, add 50 µl of DMEM to every well, except in line A to which 80 µl of DMEM should be added. This first line (A) serves as the first dilution of the assay.
- iii) For test sample titration, pipette 20 µl of each test serum to triplicate wells 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. Negative and positive reference controls of known high and low titre should be included in each test. Pipette 20 µl of each control serum into duplicate wells of the plate.

- iv) Perform serial half-dilutions of sample or reference serum, starting with row A (dilution 1/5) and proceeding down the plate, by mixing and sequentially transferring 50 µl to each subsequent row of wells. Four sera can be tested on each plate.
- v) A separate control plate should include titration of both a negative and positive (high and low) horse sera of known titre, cell control (no virus), and a back titration of virus using six wells per log dilution (100 TCID₅₀ to 0.01 TCID₅₀/well).
- vi) Prepare a virus suspension of 100 TCID₅₀ for 50 µl of DMEM. Add 50 µl of this suspension in every well with serum dilution, except those of the toxicity serum controls (column 3, 6, 9, 12) and in cell culture controls wells of control plate.
- vii) Incubate the plates at 37°C in an atmosphere with 5% CO₂ for 1 hour.
- viii) Add 100 µl of the prepared Vero cell suspension (2 × 10⁵ cells/ml) in DMEM/10% FCS to every well.
- xi) Incubate the plates at 37°C in an atmosphere with 5% CO₂ for 3 days (72 hours).
- x) The plates are examined under a microscope to observe wells for absence or presence of CPE (CPE= damaged cells). Confirm the validity of the test by establishing that the working dilution of stock virus is at 100 TCID₅₀/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 1/5 dilution. In virus controls, CPE must be present, whereas in cell controls, no CPE is found.

A positive result is considered if there is a 50% or greater reduction in the amount of viral CPE in the test serum wells compared to that in the control wells with the lowest virus dilution. The animal serum titre corresponds to the opposite of the latest dilution, for which no CPE is observed. Serum titres are comprised between 5 and 640 after multiplication of initial dilutions factors by a factor of 2; sera are indeed diluted by a factor of 2 during virus-serum mixing.

C. REQUIREMENTS FOR VACCINES

1. Background

Formalin-inactivated vaccines prepared from EEEV and WEEV propagated in cell culture against EEE and WEE viruses are commercially available. Titres obtained with inactivated vaccines might correlate with protection against EEEV and WEEV (Desanti-Consoli *et al.*, 2022). Supporting these, during the re-emergence of WEEV in South America in 2023–2024, all the reported cases corresponded to unvaccinated horses (Vissani *et al.*, 2024). EEEV and WEEV vaccines are core vaccines in the USA and attenuated EEEV and WEEV virus vaccines have not proven satisfactory.

Inactivated EEEV/WEEV vaccine in previously unvaccinated horses or of unknown vaccine history should be administered in two doses with an interval of 3–6 weeks between doses as per product label. It is recommended an annual revaccination, prior to the onset of the next vector season and annually thereafter, unless otherwise is indicated on the manufacturer's label. In animals of high risk or with limited immunity, more frequent vaccination or appropriately timed vaccination is recommended in order to induce protective immunity during periods of likely exposure (AAEP, 2025; Desanti-Consoli *et al.*, 2022). In foals, a primary two-dose series is administered beginning at 4–6 months of age with a further 4- to 6-week interval between the first and second doses. However, in foals from unvaccinated mares, the practitioner may consider starting vaccination earlier or using a product more frequently (AAEP, 2025).

The vaccines licensed for use in the USA are prepared using the following combinations: EEE and WEE; EEE, WEE, and VEE; and EEE and VEE. In addition, tetanus toxoid, inactivated influenza virus, inactivated herpes viruses (EHV-1 and EHV-4) and inactivated West Nile virus have been combined with EEE and WEE or EEE, WEE, and VEE. Current vaccines are prepared from virus propagated in cell culture, and inactivated with formalin (Maire *et al.*, 1970).

The VEEV TC-83 strain is the only licensed live-attenuated vaccine available to protect horses in endemic regions. This vaccine strain has been developed by passaging the Trinidad donkey IA/B strains 83 times in guinea-pig heart cells. The attenuation phenotype relies on two effective attenuating mutations, the first in the 5'-end untranslated region, position 3 (G > A) and the second in the E2 envelope glycoprotein, position 120 (Thr > Arg) (Go *et al.*, 2014). TC-83 is also currently used for laboratory workers and other at-risk personnel under IND status (Special

Immunization Program). However, there are substantial issues with both the immunogenicity and safety of this vaccine candidate, as TC-83 is reactogenic and poorly immunogenic in ~18%–20% of vaccinees, which raises serious safety concerns (Johnson *et al.*, 2020). An inactivated version of TC-83 (C84) is only recommended in high-risk areas. A modified live vaccine, the V3526 vaccine, was developed by introducing mutations into an infectious clone of the Trinidad donkey IAB strain and showed to be effective in protecting horses (Go *et al.*, 2014). This vaccine has several additional advantages compared with the TC-83 vaccine: it has no capacity to revert to a virulent strain, has a limited transmission potential to mosquitoes, and induces cross-protection against different viral strains. V3526 has been extensively studied in pre-clinical (animal) models, with studies showing promising results for protecting against lethal challenges of virulent VEEV strains. However, the phase I clinical trial revealed unacceptable adverse effects and development of V3526 was discontinued (Johnson *et al.*, 2020).

The recommended vaccines against VEE infection are an attenuated virus vaccine, strain TC-83, and an inactivated virus preparation made from that strain (Monath & Trent, 1981; Pan-American Health Organization, 1972; Walton, 1981; Walton & Grayson, 1989). Directions for use provided with commercial products should be followed. In contrast to EEEV and WEEV, VEEV is not currently considered a core vaccine in USA and immunisation of equines against VEEV is recommended in high-risk horses, such as those residing in US southern border states and/or horses traveling to VEE-endemic countries (AAEP, 2025). Inactivated vaccine should be administered in two doses with an interval of 2–4 weeks between doses. Annual revaccination is recommended unless otherwise is indicated on the manufacturer's label.

Attenuated VEEV vaccine should be reconstituted with physiological saline and used immediately. Multidose vials are kept on ice while the vaccine is being used. Any vaccine not used within 4 hours of reconstitution should be safely discarded. Animals over 3 months of age are vaccinated subcutaneously in the cervical region with a single dose. Annual revaccination is recommended. Recommendations specific to local environs may vary based on risk. Consultation with an equine veterinarian may be beneficial for determining the appropriate schedule.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given below 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

See chapter 1.1.8 for general requirements for Master Seeds and allowable passages for vaccine production. Suitable seed lots should be maintained at –70°C in a lyophilised state.

2.1.1. Biological characteristics of the master seed

Standard strains of EEEV and WEEV ~~viruses were~~ isolated over 20 years ago, have been used for vaccine production and have been proven to produce a protective immunity. Strains of EEEV ~~virus~~ that differ antigenically and in molecular structure have been identified from different geographical regions. However, the North American and Caribbean isolates appear to be similar (Weaver *et al.*, 1999b–1994). ~~Strains of WEE virus isolated from different countries have been found to be similar both by MAb testing and RNA oligonucleotide fingerprinting analysis (Reisen & Monath, 1989). Genetic characterisation of WEEV strains by fingerprinting analysis demonstrated that isolates from North and South America from 1941 to 1975 were remarkably homogeneous, with an estimated 90% of nucleotide identity (Weaver *et al.*, 1999b). During the re-emergence of WEEV in South America in 2023–2024, a novel WEEV lineage was identified (C lineage), closely related to the strain isolated in Argentina in 1958, by phylogenetic analysis (Campos *et al.*, 2024; Vissani *et al.*, 2024).~~ A recent well-characterised isolate from the country where the vaccine is to be used would be advantageous. Selected viruses must be immunogenic and replicate to high titres in cell culture.

The VEEV virus vaccine strain TC-83 originated from the Trinidad donkey strain (a variant of I-AB) of epizootic VEE virus isolated in 1944. This strain was derived by serial passage of the Trinidad donkey strain in fetal guinea-pig heart cells. ~~It is safe and immunogenic at the established passage levels, and induces protective immunity in vaccinated equids, although adverse reactions can sometimes occur.~~ The vaccine was originally developed for use in personnel involved in high-risk VEE virus research.

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

The MSV must be tested for purity, identity, and freedom from extraneous agents at the time before it is used in the manufacture of vaccine. The MSV must be free from bacteria, fungi and mycoplasma. The MSV is cultured on a Vero cell line and an embryonic equine cell type with confirmation by the fluorescent antibody technique to demonstrate freedom from equine herpesvirus, equine adenovirus, equine arteritis virus, bovine viral diarrhoea virus, reovirus, and rabies virus extraneous agents. The MSV must also be free from extraneous virus by cytopathic effect (CPE) and haemadsorption on cell culture on the Vero cell line and an embryonic equine cell type.

2.1.3. Validation as a vaccine strain

In an immunogenicity trial, the MSV at the highest passage level intended for production must prove its efficacy (protection) in the guinea-pig vaccination/serology potency test.

2.1.4. Emergency procedure for provisional acceptance of new master seed virus (MSV) in the case of an epizootic (with pathogens with many serotypes)

In an emergency epizootic situation, there may be not enough time to fully test a new MSV for all extraneous agents; in such a situation, provisional acceptance of the new strain could be based on a risk analysis of the possibility of contamination of the antigen produced from the new MSV with extraneous agents. This risk assessment should take into account the characteristics of the process, including the nature and concentration of the inactivant for inactivated vaccines, before allowing release of any new product.

2.2. Method of manufacture

2.2.1. Procedure

The MSV should be propagated in cell lines known to support the growth of EEEV, WEEV and VEEV ~~viruses~~. See chapter 1.1.8 for additional guidance on the preparation and testing of master cell stocks. Cell lines should be free from extraneous viruses, bacteria, fungi, and mycoplasma. Viral propagation should not exceed five passages from the MSV, unless further passages prove to provide sufficient serological titres in guinea-pigs.

The susceptible cell line is seeded into suitable vessels. Minimal essential medium, supplemented with fetal bovine serum, may be used as the medium for production. Incubation is at 37°C.

Cell cultures are inoculated directly with EEEV, WEEV or VEEV working ~~virus~~ stock, which is generally 1 to 4 passages from the MSV. Inoculated cultures are incubated for 1–3 days before harvesting the culture medium. During incubation, the cultures are observed daily for CPE and bacterial contamination.

Inactivated vaccines may be chemically inactivated with formalin and mixed with a suitable adjuvant. The duration of the inactivation period is based on demonstrated inactivation kinetics.

The preservatives used are thimerosal at a 1/1000 dilution and antibiotics (neomycin, polymyxin, amphotericin B, gentamicin, and others).

2.2.2. Requirements for ingredients

All ingredients used in the manufacture of EEEV, WEEV and VEEV vaccines should be defined in approved manufacturing protocols and consistent from batch to batch. See chapter 1.1.8 for general guidance on ingredients of animal origin. Ingredients of animal origin should be sourced from a country with negligible risk for transmissible spongiform encephalopathies (TSEs).

2.2.3. In-process controls

Production lots should be examined daily for cytopathic changes. After harvesting, the virus suspension should be tested for the presence of microbial contaminants. Production lots must be titrated in cell culture before inactivation to standardise the product. Low-titred lots may be concentrated or blended with higher-titred lots to achieve the correct titre.

Inactivated lots must be tested for completeness of inactivation in 6- to 12-hour old chicks.

2.2.4. Final product batch tests

i) Sterility

Inactivated and live vaccine samples are examined for bacterial and fungal contamination. The volume of medium used in these tests should be enough to nullify any bacteriostatic or fungistatic effects of the preservatives in the product. To test for bacteria, ten vessels, each containing a minimum of 120 ml of soybean casein digest medium, are inoculated with 0.2 ml from ten final-container samples. The ten vessels are incubated at 30–35°C for 14 days and observed for bacterial growth. To test for fungi, ten vessels, each containing a minimum of 40 ml soybean casein digest medium, are inoculated with 0.2 ml from ten final-container samples. The vessels are incubated at 20–25°C for 14 days and observed for fungal growth. Individual countries may have other requirements.

ii) Identity

Separate batch tests for identity should be conducted if the batch potency test, such as cell culture titrations of live virus vaccines, does not sufficiently verify the identity of the agent in the vaccine. Identity tests may include fluorescent antibody or serum neutralisation assays.

iii) Safety

Batch safety testing for virus inactivation is conducted in 6- to 12-hour old chicks. Ten chicks are inoculated subcutaneously with 0.5 ml of product and observed daily for 10 days. If unfavourable reactions occur, the batch is unacceptable.

iv) Batch potency

Potency testing is performed by inoculating each of ten guinea-pigs with EEEV, WEEV or VEEV-virus vaccine stock, using one-half the horse dose on two occasions, 14–21 days apart, by the route recommended for the horse. Serum samples from each vaccinee and each control are tested 14–21 days after the second dose using the PRNT-test. The EEE titres should be $\geq 1/40$, the WEE titres should be $\geq 1/40$ and the VEE titres should be $\geq 1/4$ (US Code of Federal Regulations, 2000), using Vero cells. If duck embryo fibroblasts are used in the PRNT-test, the titres will be lower. The use of animal batch release tests should be avoided wherever possible.

2.3. Requirements for regulatory approval

2.3.1. Manufacturing process

For regulatory approval of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Section 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.

In-process controls are part of the manufacturing process.

2.3.2. Safety requirements

The final inactivated vaccine formulation should be tested in a limited number of target animals prior to a larger-scale field study. The final vaccine formulation should not cause adverse reactions.

Field safety studies should be conducted before any vaccine receives final approval. Generally, two serials should be used, in three different geographical locations under typical animal husbandry conditions, and in a minimum of 600 animals. The vaccine should be administered according to label recommendations (including booster doses) and should contain the maximum permissible amount of viral antigen. (If no maximum antigen content is specified, serials should be of anticipated typical post-marketing potency.) About one-third of the animals should be at the minimum age recommended for vaccination.

i) Precautions (hazards)

Vaccine should be identified as harmless or pathogenic for vaccinators. 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

To register a commercial vaccine, a batch or batches produced according to the standard method and containing the minimum amount of antigen or potency value shall prove its efficacy (protection) in the guinea-pig vaccination/serology potency test; each future commercial batch shall be tested before release to ensure it has the same potency value demonstrated by the batch(es) used for the efficacy test(s).

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

None known.

2.3.5. Duration of immunity

Comprehensive studies on duration of immunity are not available. Current inactivated vaccines might be efficacious after a two-dose primary vaccination and yearly booster, however data supporting this statement is scarce. Moreover, studies regarding protective levels of titres against EEEV and WEEV should be further investigated and cross protection between these viruses should also be assessed. There is a clear necessity to pursue additional research on this topic. An annual revaccination is recommended for the inactivated vaccine. Foals vaccinated under 1 year of age should be revaccinated before the next vector season.

2.3.6. Stability

The inactivated vaccine is stable and immunogenic for 2 years if kept refrigerated at 2–7°C. After 2 years, vaccine should be discarded. Follow recommended expiration dates on packaging.

3. Vaccine developments

Substantial progress in alphavirus molecular virology and VEEV pathogenesis has provided a variety of powerful tools to address the safety and immunogenicity of the VEEV TC-83 vaccine. Efforts have been focused on several approaches to further improve the VEEV TC-83 vaccine with different approaches: i) infectious DNA (iDNA) technology to launch a live-attenuated vaccine *in vivo*; ii) rearrangement of alphavirus structural genes; and iii) genetic stabilisation of the TC-83-attenuated phenotype. In this way, a novel V4020 experimental vaccine was designed by securing the key TC-83 attenuating mutation (E2-T120A), along with a further improvement of attenuation, including: i) rearrangement of structural genes, ii) introduction of an additional sub-genomic promoter (26S) downstream from the glycoprotein genes; iii) synonymous translational codon replacement to secure the attenuating E2 mutation TC-83; and iv) the rescue of V4020 from a molecular clone (cDNA) to limit the population heterogeneity of the V4020 (Johnson *et al.*, 2020).

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NB: At the time of publication (2025) there were no WOA Reference Laboratories for equine encephalomyelitis (Eastern, Western and Venezuelan) (please consult the WOA Web site for the current list: <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>).

NB: EQUINE ENCEPHALOMYELITIS (EASTERN AND WESTERN) FIRST ADOPTED IN 1991; VENEZUELAN EQUINE ENCEPHALOMYELITIS FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2021.

Appendix 1.
Equine encephalomyelitis (Eastern, Western and Venezuelan):
Intended purposes of test: population freedom from infection

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>PRNT</u> <u>✓✓✓</u> <u>Equine</u>	<u>Serum</u> <u>Target: EEEV –</u> <u>WEEV - VEEV</u>	<u>Beaty <i>et al.</i> (1995)</u>	<u>Old protocols describing</u> <u>validations. However, some</u> <u>recent reported analyses have</u> <u>been carried out with VNT in</u> <u>different populations</u>	<u>Carried out by</u> <u>Johnson <i>et al.</i> (2011)</u>	<u>1. Low cost</u> <u>2. Easy to develop when</u> <u>appropriate biosafety and</u> <u>containment levels determined</u> <u>by risk analysis are available</u> <u>3. Can be performed with a</u> <u>surrogate virus under reduced</u> <u>biosafety conditions, as</u> <u>determined by biological risk</u> <u>assessment.</u> <u>4. High specificity and sensibility</u> <u>5. Provides quantitative</u> <u>information on antibody levels</u>	<u>1. In vaccinated populations:</u> <u>no differentiation between</u> <u>vaccinated and</u> <u>unvaccinated horses</u>	<u>Ni <i>et al.</i> (2007)</u> <u>Johnson <i>et al.</i></u> <u>(2011)</u> <u>Pisano <i>et al.</i> (2013)</u> <u>Juarez <i>et al.</i> (2013)</u> <u>Beaty <i>et al.</i>, 1995</u>
<u>VNT</u> <u>✓✓✓</u> <u>Equine</u>	<u>Serum</u> <u>Target: EEEV –</u> <u>WEEV - VEEV</u>	<u>Early <i>et al.</i> (1967)</u>	<u>Old protocols describing</u> <u>validations. However, some</u> <u>recent reported analyses have</u> <u>been carried out with VNT in</u> <u>different populations</u>		<u>1. Low cost</u> <u>2. Easy to develop when</u> <u>appropriate biosafety and</u> <u>containment levels determined</u> <u>by risk analysis are available</u> <u>3. Can be performed with a</u> <u>surrogate virus under reduced</u> <u>biosafety conditions, as</u> <u>determined by biological risk</u> <u>assessment.</u> <u>4. High specificity and sensibility</u> <u>5. Provides quantitative</u> <u>information on antibody levels</u>	<u>1. In vaccinated populations:</u> <u>no differentiation between</u> <u>vaccinated and</u> <u>unvaccinated horses</u>	<u>Weaver <i>et al.</i> (2014)</u> <u>Vissani <i>et al.</i>, 2024</u>

Appendix 2.
Equine encephalomyelitis (Eastern, Western and Venezuelan):
Intended purposes of test: individual animal freedom from infection prior to movement

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
RT-PCR ✓✓✓ Equine	Sample: blood Target analyte: VEEV	VEEV (Vina Rodriguez <i>et al.</i> [2016]): limit of detection for VEEV 66.2 copies/ul; for WEEV 736 copies/ul and for EEEV 316 copies/ul	VEEV: 15 synthetic RNA construct (sVEEV) representing 10 different VEEV subtypes (Vina Rodriguez <i>et al.</i> [2016])	Vina Rodriguez <i>et al.</i> (2016); Pisano <i>et al.</i> (2012)	1. Fast, sensitive and specific results 3. Easy to develop in different laboratories	1. Sample should be taken in the correct moment considering possible viremia: equids should be tested between 2 and 5 days after entering vector-protected quarantine	Pisano <i>et al.</i> (2012) Vina Rodriguez <i>et al.</i> (2016) Walton <i>et al.</i> (1973)

Appendix 3.
Equine encephalomyelitis (Eastern, Western and Venezuelan):
Intended purposes of test: confirmation of clinical cases

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>RT-PCR</u> <u>✓✓✓</u> <u>Equine</u>	<u>For target: EEEV, WEEV: CNS or other tissues</u> <u>For target VEEV: blood, CNS or other tissues</u>	<u>Nested PCR (Panalphavirus) (Sanchez Seco <i>et al.</i> [2001]): limit of detection determined as 1000 copies cDNA/first round and 1 copy/cDNA second round</u> <u>Real time:</u> <u>- NA-EEEV/WEEV (Lambert <i>et al.</i> [2003]):</u> <u>TaqMan assays 10-fold more sensitive than standard RT-PCR detecting 0.1 PFU of EEEV</u> <u>TaqMan assays 1000-fold more sensitive the standard RT-PCR detecting 0.1 PFU of WEEV</u> <u>-VEEV (Vina Rodriguez <i>et al.</i> [2016]): limit of detection for VEEV 66.2 copies/ul; for WEEV 736 copies/ul and for EEEV 316 copies/ul</u>	<u>Real time:</u> <u>NA-EEEV/WEEV: 14 isolations of NA-EEEV and 12 isolations of WEEV (Lambert <i>et al.</i> [2003])</u> <u>VEEV: 15 synthetic RNA construct (sVEEV) representing 10 different VEEV subtypes (Vina Rodriguez <i>et al.</i> [2016])</u> <u>Difficult to assess big quantities of different isolates from the three viruses</u>	<u>Carried out by Lambert <i>et al.</i> (2003); Vina Rodriguez <i>et al.</i> (2016); Pisano <i>et al.</i> (2016)</u>	<u>1. Easy to develop in different laboratories</u> <u>2. Rapid and reliable detection of the virus</u> <u>3. Low cost</u> <u>4-High sensibility and specificity</u> <u>5-Several protocols targeting different genes, including conventional or real-time PCR, pan-alphavirus and also singleplex or multiplex tests, that allow to characterise among the different viruses.</u>	<u>1. Laboratories should have capacity (equipment and trained personal) to develop the techniques</u>	<u>Sanchez-Seco <i>et al.</i> (2001)</u> <u>Johnson <i>et al.</i> (2003)</u> <u>Lambert <i>et al.</i> (2003)</u> <u>Linssen <i>et al.</i> (2000)</u> <u>Pisano <i>et al.</i> (2012)</u> <u>Vina Rodriguez <i>et al.</i> (2016)</u> <u>Vissani <i>et al.</i> (2024)</u>
<u>Virus isolation</u> <u>✓✓✓</u> <u>Equine</u>	<u>For target: EEEV, WEEV: CNS or other tissues</u> <u>For target VEEV: blood, CNS or tissues</u>	<u>Early <i>et al.</i> (1967)</u> <u>Beaty <i>et al.</i> (1995)</u>	<u>Virus isolation was performed in several outbreaks and few experimental infections</u>	<u>See references</u>	<u>1. Low cost</u> <u>2. Easy to develop when appropriate biosafety and containment levels determined by risk analysis</u>	<u>1. Need for appropriate biosafety and containment levels determined by risk analysis</u> <u>2. Can take up to 1 week – time-consuming</u> <u>3. Samples should be maintained in correct conditions</u>	<u>Del Piero <i>et al.</i> (2001)</u> <u>Weaver <i>et al.</i> (2012)</u> <u>Logue <i>et al.</i>, (2009)</u> <u>Vissani <i>et al.</i>, (2024)</u>

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>In-situ hybridisation</u> ☒ ☐ ☐ <u>Equine</u>	<u>Sample: paraffin-embedded of tissues (CNS and others)</u> <u>Target: EEEV and WEEV</u>	<u>For WEEV: Representative sections from different part of CNS and other tissues from five horses have been analysed by ISH and compared with Ct of the same tissues by RT-PCR. Positive results of RT-PCR from the five horses were confirmed by ISH. Quantitative analysis correlate WEEV-ISH specific with RT-PCR. Both ISH and Ct values from RT-PCR were significantly inversely correlated ($p = 0.0149$) indicating positive correlation between WEEV RNA ISH signal and viral genomic RNA load by RT-PCR.</u> <u>For EEEV: A two-tiered Fisher exact test confirmed that there was no significant deviation between the ability for ISH to detect EEEV and antigens using RT-PCR as the gold standard for a positive result ($p = 1.00$ for EEEV).</u>	<u>See references</u>	<u>See references</u>	<u>1. Can be used with CNS or tissues that are not acceptable for isolation/RT-PCR</u> <u>2. Probes designed specifically to differentiate WEEV from EEEV</u> <u>3. No need for appropriate biosafety and containment levels determined by risk analysis</u>	<u>1. Failure to identify viral antigen in equine CNS by ISH does not rule out infection</u> <u>2. Need for specialised equipment not available in all diagnostic laboratories.</u>	<u>Vissani <i>et al.</i> (2024)</u> <u>Pennick <i>et al.</i> (2012)</u>
<u>Whole genome sequencing</u> ☒ ☒ ☒ <u>Equines</u>	<u>Sample: blood – CNS or other tissues</u> <u>Target: VEEV</u>	<u>The array correctly classified 76 out of 84 cDNA samples. Four of the 8 misclassified cases were highly similar sequences collected in the same location. The overall concordance rate was 96.2%.</u>	<u>Panel of 133 VEEV isolates has been evaluated</u>	<u>See references</u>	<u>1. Rapid genomic evaluation of the strain from an epizootic outbreak event</u> <u>2. Reduced need for viral isolation and culturing, allowing viruses to be characterised directly from a tissue sample</u>	<u>1. Need for specialised equipment and trained personnel: available in few laboratories</u> <u>2. Expensive</u>	<u>Gardner <i>et al.</i> (2016)</u> <u>Moncayo <i>et al.</i> (2001)</u>
<u>PRNT</u> ☒ ☒ ☐ <u>Equine</u>	<u>Serum (paired).</u> <u>Target: EEEV, WEEV and VEEV</u>	<u>Beatty <i>et al.</i> (1995)</u>	<u>Old protocols describing validations. A few recent reported analyses have been</u>	<u>See references</u>	<u>1. Low cost</u> <u>2. Easy to develop when appropriate biosafety and containment levels determined by risk analysis are available</u>	<u>1. For EEEV and VEEV, horses died before getting the paired sample</u> <u>2. Cannot differentiate</u>	<u>Ni <i>et al.</i> (2007)</u> <u>Johnson <i>et al.</i> (2011)</u>

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
			<u>carried out with PRNT in different populations</u>		<u>3. Can be performed with a surrogate virus under reduced biosafety conditions, as determined by biological risk assessment</u> <u>4. High specificity and sensibility</u> <u>5. Provides quantitative information on antibody levels</u>	<u>between vaccinated and unvaccinated horses</u>	<u>Pisano <i>et al.</i> (2013)</u> <u>Juarez <i>et al.</i> (2013)</u>
<u>VNT</u> ☒ ☒ ☐ <u>Equine</u>	<u>Serum</u> <u>Target: EEEV, WEEV and VEEV</u>	<u>Early <i>et al.</i> (1967)</u>	<u>Old protocols describing validations A few recent reported analyses have been carried out with VNT in different populations</u>	<u>See references</u>	<u>1. Low cost</u> <u>2. Easy to develop when appropriate biosafety and containment levels determined by risk analysis are available</u> <u>3. Can be performed with a surrogate virus under reduced biosafety conditions, as determined by biological risk assessment</u> <u>4. High specificity and sensibility</u> <u>5. Provides quantitative information on antibody levels</u>	<u>1. For EEEV and VEEV, horses died before getting the paired sample</u> <u>2. Cannot differentiate between vaccinated and unvaccinated horses</u>	<u>Vissani <i>et al.</i> (2024)</u> <u>Weaver <i>et al.</i> (2014)</u>

Appendix 4.
Equine encephalomyelitis (Eastern, Western and Venezuelan):
Intended purposes of test: prevalence of infection – surveillance

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>PRNT</u> <u>✓✓✓</u> <u>Equine</u>	<u>Serum</u> <u>Target: EEEV,</u> <u>WEEV, VEEV</u>	<u>Beaty <i>et al.</i> (1995)</u>	<u>Old protocols describing validations. A few recent reported analyses have been carried out with PRNT in different populations</u>	<u>See references</u>	<u>1. Low cost</u> <u>2. Easy to develop when appropriate biosafety and containment levels determined by risk analysis are available</u> <u>3. Can be performed with a surrogate virus under reduced biosafety conditions, as determined by biological risk assessment</u> <u>4. High specificity and sensibility</u> <u>5. Provides quantitative information on antibody levels</u>	<u>1. No differentiation between vaccinated and unvaccinated horses</u>	<u>Ni <i>et al.</i> (2007)</u> <u>Johnson <i>et al.</i> (2011)</u> <u>Pisano <i>et al.</i> (2013)</u> <u>Juarez <i>et al.</i> (2013)</u>
<u>VNT</u> <u>✓✓✓</u> <u>Equine</u>	<u>Serum</u> <u>Target: EEEV,</u> <u>WEEV, VEEV</u>	<u>Early <i>et al.</i> (1967)</u>	<u>Old protocols describing validations. A few recent reported analyses have been carried out with VNT in different populations</u>	<u>See references</u>	<u>1. Low cost</u> <u>2. Easy to develop when appropriate biosafety and containment levels determined by risk analysis are available</u> <u>3. Can be performed with a surrogate virus under reduced biosafety conditions, as determined by biological risk assessment</u> <u>4. High specificity and sensibility</u> <u>5. Provides quantitative information on antibody levels</u>	<u>1. No differentiation between vaccinated and unvaccinated horses</u>	<u>Vissani <i>et al.</i> (2024)</u> <u>Weaver <i>et al.</i> (2014)</u>

Appendix 5.
Equine encephalomyelitis (Eastern, Western and Venezuelan):
Intended purposes of test: immune status in individual animals or populations post-vaccination

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>PRNT</u> <u>✓✓✓</u> <u>Equine</u>	<u>Serum</u> <u>Target: EEEV,</u> <u>WEEV, VEEV</u>	<u>Beaty <i>et al.</i> (1995)</u>	<u>Old protocols describing the technique. A few recent reported analyses have been carried out with PRNT in different populations</u>	<u>See references</u>	<u>1. Low cost</u> <u>2. Easy to develop when appropriate biosafety and containment levels determined by risk analysis are available</u> <u>3. Can be performed with a surrogate virus under reduced biosafety conditions, as determined by biological risk assessment</u> <u>4. High specificity and sensibility</u> <u>5. Provides quantitative information on antibody levels</u>	<u>1. No differentiation between vaccinated and unvaccinated horses</u>	<u>Ni <i>et al.</i> (2007)</u> <u>Johnson <i>et al.</i> (2011)</u> <u>Pisano <i>et al.</i> (2013)</u> <u>Juarez <i>et al.</i> (2013)</u>
<u>VNT</u> <u>✓✓✓</u> <u>Equine</u>	<u>Serum</u> <u>Target: EEEV,</u> <u>WEEV, VEEV</u>	<u>Early <i>et al.</i> (1967)</u>	<u>Old protocols describing validations. A few recent reported analyses have been carried out with VNT in different populations</u>	<u>See references</u>	<u>1. Low cost</u> <u>2. Easy to develop when appropriate biosafety and containment levels determined by risk analysis are available</u> <u>3. Can be performed with a surrogate virus under reduced biosafety conditions, as determined by biological risk assessment</u> <u>4. High specificity and sensibility</u> <u>5. Provides quantitative information on antibody levels</u>	<u>1. No differentiation between vaccinated and unvaccinated horses</u>	<u>Vissani <i>et al.</i> (2024)</u> <u>Weaver <i>et al.</i> (2014)</u>

MEETING OF THE BIOLOGICAL STANDARDS COMMISSION

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CHAPTER 3.6.6.

EQUINE INFLUENZA
(INFECTION WITH EQUINE INFLUENZA VIRUS)

SUMMARY

Description of the disease: Equine influenza is an acute respiratory infection of horses, donkeys, mules and zebras caused by two distinct subtypes (H7N7, formerly equi-1, and H3N8, formerly equi-2) of influenza A virus within the genus *Alphainfluenzavirus A* of the family Orthomyxoviridae. Viruses of the H7N7 subtype have not been isolated since the late 1970s. Equine influenza viruses of both subtypes are considered to be of avian ancestry and highly pathogenic avian H5N1 has been associated with an outbreak of respiratory disease in donkeys in Egypt. In fully susceptible equidae, clinical signs include pyrexia and a harsh dry cough followed by a mucopurulent nasal discharge. In partially immune vaccinated animals, one or more of these signs may be absent. Vaccinated infected horses can still shed the virus and serve as a source of virus to their cohorts. Characteristically, influenza spreads rapidly in a susceptible population. The disease is endemic in many countries with substantial equine populations.

While normally confined to equidae, equine H3N8 influenza has crossed the species barrier to dogs. Extensive infection of dogs has been reported in North America where it normally produces mild fever and coughing but can cause fatal pneumonia. While equine influenza has not been shown to cause disease in humans, serological evidence of infection has been described primarily in individuals with an occupational exposure to the virus. During 2004–2006 influenza surveillance in central China (People's Rep. of) two equine H3N8 influenza viruses were also isolated from pigs.

Identification of the agent: Embryonated hens' eggs and/or cell cultures can be used for virus isolation from nasopharyngeal swabs or nasal and tracheal washes. Isolates should always be sent immediately to a WOA Reference Laboratory. Infection may also be demonstrated by detection of viral nucleic acid ~~or antigen in respiratory secretions using the~~ reverse-transcription polymerase chain reaction (RT-PCR) or detection of antigen in respiratory secretions using an antigen-capture enzyme-linked immunosorbent assay (ELISA) or a rapid antigen-detection test kit respectively.

Serological tests: Diagnosis of influenza virus infections is usually only accomplished by tests on paired sera; the first sample should be taken as soon as possible after the onset of clinical signs and the second approximately 2–4 weeks later. Antibody levels are determined by haemagglutination inhibition (HI) ~~or~~ single radial haemolysis (SRH) or-ELISAs.

Requirements for vaccines: Spread of infection and severity of disease may be reduced by the use of potent ~~inactivated~~ equine influenza vaccines containing epidemiologically relevant virus strains. Inactivated equine influenza vaccines contain whole viruses or their subunits. The vaccine viruses are propagated in embryonated hens' eggs or tissue culture, concentrated, and purified before inactivation with agents such as formalin or beta-propiolactone. Inactivated vaccines provide protection by inducing humoral antibody to the haemagglutinin protein. Responses are generally short-lived and multiple doses are required to maintain protective levels of antibody. An adjuvant is usually required to stimulate durable protective levels of antibody. Vaccines containing recombinant canarypox viruses expressing the haemagglutinin (HA) gene from equine influenza virus are widely used, and a nucleoprotein-based ELISA can differentiate infected animals from

vaccinates (DIVA). A live attenuated virus and viral vectored vaccines have been licensed in some countries.

Vaccine breakdown has been attributed to inadequate vaccine potency, inappropriate vaccination schedules, and outdated vaccine viruses that are compromised as a result of antigenic drift. ~~An in-vitro potency test (single radial diffusion) can be used for in-process testing of the antigenic content of inactivated products before addition of an adjuvant. In-process testing of live and vectored vaccines relies on titration of infectious virus.~~ International surveillance programmes monitor antigenic drift among equine influenza viruses and each year the Expert Surveillance Panel (ESP) for Equine Influenza makes recommendations for suitable vaccine strains. Following a change in recommendations, vaccines should be updated as quickly as possible to ensure optimal protection. This is particularly important for highly mobile horse populations and for any horse travelling internationally.

A. INTRODUCTION

Equine influenza is caused by two subtypes: H7N7 (formerly subtype 1) and H3N8 (formerly subtype 2) of influenza A viruses (genus Alphainfluenzavirus A of the family Orthomyxoviridae); however there have been very few reports of H7N7 subtype virus infections in the last ~~30-40~~ years (~~Webster, 1993~~). As Equine H7N7 viruses are considered extinct, it is no longer necessary to include a representative of this subtype in vaccines.

In fully susceptible equidae, clinical signs include pyrexia, nasal discharge and a harsh dry cough; pneumonia in young foals and donkeys and encephalitis in horses have been described as rare events (~~Daly et al., 2006; Gerber, 1970~~). High fatality rates may occur in naïve equidae (Diallo et al., 2021). ~~Clinical signs associated with infection in dogs also include fever and a cough; occasionally infection results in suppurative bronchopneumonia and peracute death (Crawford et al., 2005).~~ Characteristically, influenza spreads rapidly in a susceptible population. The virus is spread by the respiratory route, and indirectly by contaminated personnel, vehicles and fomites. The incubation period in susceptible horses may be less than 24 hours.

In partially immune vaccinated animals the incubation period may be extended, one or more clinical signs may be absent and spread of the disease may be limited. This makes clinical diagnosis of equine influenza more difficult as other viral diseases, such as equine herpesvirus-associated respiratory disease, may clinically resemble a mild form of influenza. Horses infected with equine influenza virus become susceptible to secondary bacterial infection and may develop mucopurulent nasal discharge, which can lead to diagnosis of bacterial disease with the underlying cause being overlooked.

Equine influenza viruses are believed to be of avian ancestry, and more recent transmission of avian viruses to horses and donkeys has been recorded. The sequence analysis of an H3N8 virus isolated in 1989 from horses during a limited influenza epidemic in northeastern China (People's Rep. of) established that the virus was more closely related to avian influenza viruses than to equine influenza viruses (~~Guo et al., 1992~~). Avian H5N1 has been associated with respiratory disease of donkeys in Egypt (~~Abdel Moneim et al., 2010 reviewed by Chambers 2022~~).

Equine influenza viruses have the potential to cross species barriers and have been associated with respiratory disease in dogs primarily in North America (~~Crawford et al., 2005~~). This H3N8 canine influenza virus was geographically restricted to North America and has become extinct (Wasik et al., 2023). Isolated outbreaks of equine influenza ~~have~~ also occurred in dogs within the UK, but the virus has not become established in the canine population. Clinical signs associated with infection in dogs also include fever and a cough; occasionally infection results in suppurative bronchopneumonia and peracute death. ~~Close~~ Contact with infected horses was thought to be involved in each outbreak in the UK. Equine influenza viruses have also been isolated from pigs in central China (People's Rep. of) (Tu et al., 2009). Despite the occasional identification of seropositive persons with occupational exposure there is currently little evidence of zoonotic infection of people with equine influenza (~~Alexander & Brown, 2000~~).

In endemic countries the economic losses due to equine influenza can be minimised by vaccination and many racing authorities and equestrian bodies have mandatory vaccination policies. Vaccination does not produce sterile immunity; vaccinated horses may shed virus and contribute silently to the spread of the disease. Appropriate risk management strategies to deal with this possibility should be developed.

For recent information on distribution at the country level please consult the WAHIS interface (<https://wahis.woah.org/#/home>).

B. DIAGNOSTIC TECHNIQUES

Test methods available for the diagnosis of equine influenza and their purpose are summarised in Table 1. Laboratory diagnosis of acute equine influenza virus infections is based on virus detection in nasal or nasopharyngeal swabs collected from horses with acute respiratory illness. Alternatively, the Nasopharyngeal swabs have been shown under experimental conditions, to display a higher detection frequency and viral load compared with nasal swabs (Paillot *et al.*, 2013). Real-time reverse-transcription polymerase chain reaction (RT-PCR) for the demonstration-detection of a serological response to infection may be attempted with paired serum samples. Ideally, both methods are used. Equine influenza virus may be isolated-viral genome is the frontline test in embryonated hens' eggs or cell culture-the majority of diagnostic laboratories. Infection may also be demonstrated by detection of viral antigen in respiratory secretions using an antigen capture enzyme-linked immunosorbent assay (ELISA) or of viral genome using reverse transcription polymerase chain reaction (RT-PCR) assays, or a rapid antigen-detection test kit. Equine influenza virus may be isolated in embryonated hens' eggs or cell culture. As a diagnostic technique, virus isolation has been replaced by more rapid virus-detection assays but it is necessary for antigenic characterisation and strain surveillance. Demonstration of a serological response to infection may be attempted with paired serum samples.

All influenza viruses are highly contagious for susceptible hosts and care must therefore be taken during the handling of infected eggs or cultures to avoid accidental cross-contamination. Standard strains should not be propagated in the diagnostic laboratory, at least never at the same time or in the same place where diagnostic samples are being processed. All working areas must be efficiently disinfected before and after virus manipulations, which should preferably be conducted within biohazard containment level 2 and class II safety cabinets.

It is important to obtain samples as soon as possible after the onset of clinical signs, preferably within 3–5 days. These samples include nasopharyngeal swabs and nasal or tracheal washings, the latter taken by endoscopy. Swabs may consist of absorbent cotton wool sponge/gauze on wire, and should be long enough to be passed via the ventral meatus into the nasopharynx. Swabs should be transferred to a tube containing transport medium immediately after use. This medium consists of is prepared in 500 ml volumes and includes 470 ml of sterile phosphate buffered saline (PBS) containing either 40% glycerol or 2% tryptose phosphate broth with 2% antibiotic solution (penicillin [10,000 units], streptomycin [10,000 units] in sterile distilled water [100 ml]), and 2% fungizone (250 mg/ml stock), 10 ml penicillin/streptomycin (penicillin 5000 IU/ml, streptomycin 5000 mcg/ml), 10 ml amphotericin b (250 mg/ml) and 10 ml of heat-inactivated fetal bovine serum or 10 ml tryptose phosphate broth (29.5 g/litre). The medium is aliquoted in 5 ml quantities and stored at –18°C to –26°C. If viral transport medium is not available, sterile normal saline solution will serve an immediate need although such samples should be inoculated as soon as possible. Before being frozen, antibiotics and fetal bovine serum/tryptose phosphate broth should be added as above.

If the samples are to be inoculated within 1–2 days they may be held at 4°C, but, if kept for longer, they should be stored at –70°C or below. Samples should be kept cool during transport to the laboratory.

Sample processing should follow the quality procedures outlined in Chapter 1.1.5 *Quality management in veterinary testing laboratories*, taking measures to prevent cross contamination. The liquid is expelled from the swab by squeezing with forceps and the swab is then disposed of suitably. Further antibiotics may be added if samples appear to be heavily contaminated with bacteria. The remainder of the fluid is stored at –70°C. Samples treated with antibiotics are allowed to stand on ice for 30–60 minutes and are then centrifuged at 1500 g for 15 minutes to remove bacteria and debris; the supernatant fluids are used for inoculation. The remainder of the fluid is stored at –70°C. Filtration of samples is not advised as influenza virus may adsorb on to the filter and be lost from the sample.

Table 1. Test methods available for the diagnosis of equine influenza 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,e)	Prevalence of infection – surveillance ^(e)	Immune status in individual animals or populations post-vaccination ^(f)
Identification of the agent^(a)						
Virus isolation	□□□	☑□□	□□□	☑☑□	□□□	□□□
Real-time RT-PCR	☑☑☑ □□□	☑☑☑	☑☑☑	☑☑☑	☑☑☑ ☑☑☑	□□□

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, e)	Prevalence of infection – surveillance ^(e)	Immune status in individual animals or populations post-vaccination ^(f)
RAD	☑☐☐ ☐☐☐	☑☐☐	☐☐☐	☑☑☐	☑☐☐	☐☐☐
Antigen-capture ELISA	☑☐☐ ☐☐☐	☑☐☐ ☑☑☐	☐☐☐ ☑☑☐	☑☐☐ ☑☑☐	☑☐☐ ☑☑☐	☐☐☐
Detection of immune response						
HI	☑☑☐	☑☑☐ ^(g-b)	☐☐☐	☑☑☐ ☑☑☐ ^(g-b)	☑☑☐ ☑☑☐	☑☑☐
SRH	☑☑☐	☑☑☐ ^(g-b)	☐☐☐	☑☑☐ ☑☑☐ ^(g-b)	☑☑☐ ☑☑☐	☑☑☐
ELISA	☑☑☐	☑☐☐ ^(h)	☑☑☐ ^(h-e)	☑☐☐ ^(g-b)	☑☑☐ ☑☑☐	☑☐☐

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; RAD = rapid antigen detection;

HI = haemagglutination inhibition; SRH = single radial haemolysis; ELISA = enzyme-linked immunosorbent assay.

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

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

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

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

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

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

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

^(g-b)Testing of paired samples required;

^(h-e)May be useful for DIVA (detection of infection in vaccinated animals) when used with appropriate vaccines.

1. Identification of the agent

Isolation of infectious virus may be carried out in embryonated hens' eggs or cell cultures. Traditionally, eggs have been preferred for isolation of equine influenza as many clinical isolates do not grow well in cells without serial passage. Comparison of H3N8 viruses isolated in eggs and Madin–Darby canine kidney (MDCK) cells indicated that MDCK cells are capable of selecting variant viruses that are not representative of the predominant virus in clinical specimens (Ilobi *et al.*, 1994). However, some viruses have been successfully isolated in MDCK cells but not in eggs and selection of variants has also occurred as a result of culture in eggs (Oxburgh & Klingborn, 1999). Ideally, isolation should be attempted using both substrates.

RT-PCR and real-time RT-PCR assays are being widely used in diagnostic laboratories as a more sensitive alternative to virus isolation (Quinlivan *et al.*, 2005; Galvin *et al.*, 2014; Gildea *et al.*, 2018). Influenza virus antigen in nasal secretions may also be detected directly by ELISA or rapid antigen-detection kits ~~a sensitive antigen-capture ELISA for the H3N8 virus using a monoclonal antibody (MAb) against the equine influenza virus nucleoprotein (Livesay *et al.*, 1993). This assay is not commercially available, other than as a diagnostic service, but commercial self-contained kits for detecting human influenza are available and have been shown to detect equine influenza antigen (Chambers *et al.*, 1994; Galvin *et al.*, 2014; Yamanaka *et al.*, 2008–2016).~~ This approach is less sensitive than RT-PCR but provides a rapid result on which preliminary management decisions may be based. ~~These techniques~~ should not be used to the exclusion of virus isolation. It is essential that new viruses be isolated and sent to reference laboratories for characterisation as part of the surveillance programme to monitor antigenic drift and emergence of new viruses and to provide isolates for inclusion in updated vaccines. Positive RT-PCR and ELISA results are useful in the selection of samples for virus isolation attempts if resources are limited, or for the selection of specimens to be sent to a reference laboratory for virus isolation and characterisation.

1.1. Virus isolation in embryonated hens' eggs

Fertile eggs are set in a humid incubator at 37–38°C and turned twice daily; after 10–11 days, they are examined by candling and live embryonated eggs are selected for use. The area above the air sac is cleansed with alcohol and a small hole is made through the shell. Inoculum may be introduced into the amniotic or the allantoic cavity. Several eggs/sample are inoculated (0.1 ml) in the amniotic cavity with no additional dilution of the sample (sample may also be diluted 1/10 and 1/100 in PBS

containing antibiotics). The syringe is withdrawn approximately 1 cm and a further 0.1 ml is inoculated into the allantoic cavity. Alternatively, many laboratories opt to inoculate into the allantoic cavity alone through a second hole drilled just below the line of the air sac. The hole(s) is/are sealed with wax or Sellotape, and the eggs are incubated at 34–35°C for 3 days. The embryos that die within 24 hours following inoculation should be discarded. The eggs that contain embryos that die more than 24 hours after inoculation or contain live embryos after 3 days are examined for the presence of equine influenza virus.

Following incubation, the eggs are transferred to 4°C for 4 hours or overnight to kill the embryos and to reduce bleeding at harvest. The shells are disinfected, and the amniotic and/or allantoic fluid is harvested by pipette, each harvest being kept separate. These are tested for haemagglutination (HA) activity by mixing twofold dilutions of the harvested fluid in equal volumes (0.025 ml) with chicken red blood cells (RBCs) (0.5%–1% [v/v] packed cells in PBS or PBS with 0.05% bovine serum albumin (BSA) solution¹) in V- or U-bottomed microtitre plates or 0.4% guinea-pig RBCs (0.4% [v/v] packed cells in PBS) in V- or U-bottomed plates. Plates are incubated for approximately 30 minutes preferably at 4°C to prevent neuraminidase activity. ~~If chicken RBCs are used, The plates may be read by tilting to 70° so that non-agglutinated cells 'stream' to the bottom of the well. Non-agglutinated guinea-pig cells appear as a button at the bottom of the well and may take longer to settle.~~ If there is no HA activity, aliquots of each harvest are pooled and passaged into further eggs. All HA positive samples are divided into aliquots and stored at –70°C; one aliquot is titrated for HA immediately. The HA titre is the reciprocal of the greatest dilution to show agglutination. If the HA titre is 1/16 or more, the isolate is characterised immediately. If titres are low, positive samples should be passaged. Care should be taken to avoid generation of defective interfering particles by prediluting the inoculum 1/10, 1/100, 1/1,000. Positive samples arising from the highest dilution should be selected as stocks for storage. It may be necessary to undertake as many as five passages to isolate the virus, particularly from vaccinated horses. If virus has not been recovered by the fifth passage, further passages are unlikely to be successful.

1.2. Virus isolation in cell cultures

Cultures of the MDCK cell line (MDCK, ATCC CCL34) may be used to isolate equine influenza viruses. The cells are grown to confluence in tubes and then infected in triplicate with 0.25–0.5 ml of each sample, processed as described above. Prior to inoculation, the cell monolayer is washed at least once with tissue culture medium containing trypsin (2 µg/ml) without serum. The cultures are maintained with serum-free medium containing 0.5–2 µg/ml trypsin (treated with TPCK [L-1-tosylamine-2-phenylethyl chloromethyl ketone] to remove chymotrypsin, available pretreated, e.g. from Sigma), and examined daily for evidence of cytopathic effects (CPE). If positive, or after 7 days in any case, the supernatant fluids are tested for HA. Fluids with titres of ≥1/16 are characterised immediately. Negative fluids and those with titres <1/16 are repassaged up to five passages.

Alternatively, the cells are screened for evidence of haemadsorption (HAD). This procedure detects expression of viral antigens at the cell surface. The medium is removed from the cultures and the tubes are washed with PBS. One or two drops of a 50% suspension of chicken or guinea-pig RBCs are added, the tubes are rotated carefully, and kept at room temperature (23°C ± 2°C) for 30 minutes. Unbound RBCs are washed off with PBS, and the cultures are examined microscopically for evidence of HAD.

1.3. Haemagglutinin subtyping characterisation

The HA subtype of ~~new~~ isolates of equine influenza viruses may be determined by haemagglutination inhibition (HI; Section B.2.1) using H7N7- and H3N8-specific antisera. ~~Isolates may first be treated with Tween 80/other, which destroys viral infectivity and reduces the risk of cross-contamination. In the case of H3N8 viruses particularly, this treatment enhances the HA activity (John & Fulginiti, 1966). However, treatment with Tween 80/other also decreases specificity and may increase the variability of the results obtained. Standard antigens must be titrated in parallel with tests to identify viruses and should include H7N7 strains (e.g. A/eq/Prague/56, A/eq/Newmarket/77) and H3N8 strains (e.g. A/eq/Newmarket/2/03, and A/eq/South Africa/4/03 and A/eq/Richmond/1/07). Virus strains may be obtained from WOAHP Reference Laboratories (see Table given in Part 4 of this Terrestrial Manual). Additionally, recent isolates from the same geographical area should be included if available. The standard antigens should be treated with Tween 80/other to avoid cross-contamination. Test antigens and standard antigens are always back-titrated to confirm their antigen content.~~

Since the 1980s, only subtype H3N8 viruses have been isolated from horses. ~~The HA sequence of equine H3 isolates can be determined rapidly by RT-PCR and sequencing, as described by Rash et~~

¹ Stock used is 30% ± 2% BSA solution in 0.85% NaCl.

~~al. (2014), and is encouraged for surveillance purposes.~~ New isolates of equine influenza viruses may be further characterised by HI using ~~strain~~ clade-specific antisera. The species in which antibodies are raised will influence the cross-reactivity of the antiserum, with ferrets providing the most strain-specific antibody (~~Mumford, 1992~~). The specificity and cross reactivity of the sera are also influenced by the immunisation schedule. Sera obtained 3 weeks after a single antigen application are considered to be most discriminative. Virus strains and clade specific ferret antisera may be obtained from WOA reference laboratories for the purpose of antigenic characterisation.

The HA sequence of equine H3 isolates can be determined rapidly by RT-PCR and sequencing, as described by Rash *et al.* (2014), and is encouraged for surveillance purposes. All isolates should be sent immediately to an International Reference Laboratory designated by WOA or ~~the World Health Organization (WHO)~~ for inclusion in the strain surveillance programme to monitor antigenic drift and emergence of new viruses.

1.4. Neuraminidase ~~subtyping~~ characterisation

~~Subtyping of neuraminidase requires specific antisera and no routine technique is available. Subtyping can also be done using specific PCR primers. Since the 1980s, only subtype H3N8 viruses have been isolated from horses. The~~ In common with approaches for HA determination, the NA sequence of equine H3 isolates can be determined rapidly by RT-PCR and sequencing, as described by Rash *et al.* (2014), and is encouraged for surveillance purposes.

1.5. Real-time reverse transcriptase polymerase chain reaction

Real-time RT-PCR assays have replaced both conventional assays and real-time, and are routinely used for the detection of equine influenza genome in nasal secretions as they are more sensitive than virus culture in eggs or detection of nucleoprotein using rapid antigen-detection (RAD) kits or ELISA for the detection of human influenza. A probe-based real-time RT-PCR assay based on the matrix gene and developed for the detection of a wide range of influenza type A strains including avian H5N1 (Heine *et al.*, 2007) was combined with an automated DNA extraction system to establish a high throughput assay used in the mass screening of horses during the eradication programme in Australia in 2007 (~~Foord *et al.*, 2009~~). This type of paninfluenza assay has been used effectively for diagnosis and surveillance of equine influenza by WOA Reference Laboratories (Gildea *et al.*, 2018). Real-time RT-PCR assays based on NP, M, and HA genes of H3N8 and H7N7 subtypes of equine influenza have been described (Lu *et al.*, 2009) as have specific for equine-2 influenza (H3N8) and equine-1 (H7N7) viruses have also been described assays that distinguish between clade 1 and clade 2 of the Florida sublineage of H3N8 (Briser *et al.*, 2019), and a Commercial real-time RT-PCR kits for the detection of equine influenza has become are also available in some countries (Lu *et al.*, 2009).

The WOA Reference Laboratories selected a pan-reactive influenza type A real-time RT-PCR assay targeting the matrix gene based on the assay described by Heine *et al.* (2007) for validation in line with the WOA Validation Template. The assay has been validated for the intended purpose of certifying freedom from infection in individual animals for trade or movement.

Table 2. Primers and probe sequences for a pan-reactive influenza type A real-time RT-PCR

<u>Pathogen/ target gene</u>	<u>Primer^(a)/probe (5'–3')</u>	<u>Concentration</u>	<u>Cycling parameters^(b)</u>
<u>Heine <i>et al.</i>, 2007</u>			
<u>Matrix</u>	<u>Type A Fwd: AGA-TGA-GYC-TTC-TAA-CCG-AGG-TCG</u> <u>Type A Rev: TGC-AAA-NAC-ATC-YTC-AAG-TCT-CTG</u> <u>Type A Probe: 6-FAM-TCA-GGC-CCC-CTC-AAA-GCC-GA-</u> <u>TAMRA</u>	<u>320 nM</u> <u>320 nM</u> <u>80 nM</u>	<u>40 cycles of: 95°C/15 sec</u> <u>and 60°C/45 sec</u>

^(a)N=A, G, C or T and Y=C or T. ^(b)The reverse transcription and denaturation steps prior to cycling have not been included (see below).

The sequences of the PCR primers used are:

Forward: 5'-AGA-TGA-GYC-TTC-TAA-CCG-AGG-TCG-3'

Reverse: 5'-TGC-AAA-NAC-ATC-YTC-AAG-TCT-CTG-3'

Probe: 6-FAM-5'-TCA-GGC-CCC-CTC-AAA-GCC-GA-3'-TAMRA

N=A, G, C or T and Y=C or T

The protocol below may require modification to accommodate individual laboratory or different RT-PCR kit requirements. Strong positive, weak positive and negative reference samples are available from the WOA Reference Laboratory at the Irish Equine Centre to facilitate the setting up, validation and monitoring of the assay.

- i) RNA is extracted from ~~400-200~~ 100-200 µl nasopharyngeal secretions. Nucleic acid is eluted in ~~80-90~~ 40-60 µl of elution buffer and 5 µl is used as the template in each 25 µl one-step RT-PCR reaction.

Commercial extraction kits are widely available; the viral nucleic acid extraction step can be performed according to the procedures specified in each kit. A synthetic internal positive control is introduced at this stage and is included throughout the process to monitor for PCR inhibitors, i.e. to reduce the likelihood of false negatives.

- ii) Kits for the one-step real-time RT-PCR are available commercially. Below are some basic steps that can be modified depending on local requirements, kits used and equipment available.
- iii) Primers and probes are stored at a working dilution of 10 µM.
- iv) An appropriate volume of real-time one-step RT-PCR master mix for the number of samples to be tested is prepared, following the manufacturer's instructions. Typically, 20 µl per sample consists of 12.5 µl 2 × RT buffer, 2.0 µl tRNA, 0.8 µl of each primer (final concentration 0.32 µM), 0.2 µl of probe (final concentration 0.08 µM), 1.0 µl 25 × RT-PCR enzyme mix, ~~4.25-0.6~~ 4.25-0.6 µl IPC (internal positive control) primer/probe mix and ~~4.45-2.1~~ 4.45-2.1 µl nuclease free water.
- v) 5 µl of RNA sample including test and positive and negative controls, are added to 20 µl of the master mix in the appropriate wells of the PCR plate.
- vi) The plate is placed in a real-time thermal cycler programmed for reverse transcription and cDNA amplification or fluorescence detection as suggested by the manufacturers. The following thermal profile is an example: reverse transcription at 45°C for 10 minutes followed by denaturation at 95°C for 10 minutes. cDNA is amplified by 40 cycles of 95°C for 15 seconds and 60°C for 45 seconds.

Note: times and temperatures may vary and should be optimised for the reagents or kit used.

1.5.1. Interpretation of the results

The results are expressed at cycle threshold (Ct) values representing the number of cycles necessary for a statistically significant rise in reporter dye emission. A threshold bar used to determine these values is set manually to a consistent ΔR_n value about halfway along the linear portion of an amplification plot. Ct values up to 40 are considered positive. Samples are classified as negative if the IPC Ct is within the range acceptable to the manufacturer and the Ct for influenza is 40 or above.

1.6. Molecular characterisation

The real-time RT-PCR assay outlined above is not specific to equine influenza virus and will also detect avian and swine influenza viruses. It is possible that it will also detect human influenza viruses that may incidentally contaminate either the equine nasopharynx or the sample, although it is considered that the risk of this is very low. To further investigate any samples that test positive by RT-PCR and to monitor genetic evolution, attempts should be made to sequence the HA – it is necessary to determine the sequences of HA and NA genes. It is preferable to sequence from the nasal swab directly. The reliability and quality of data reduces with increasing cycle threshold values in samples from real-time RT-PCR testing, i.e. low viral load. Sequencing of the HA and NA is traditionally performed by Sanger sequencing using several segment-specific primers. The assay described below is based on that described by Rash *et al.* (2014) and has been used for both Florida clade 1 and Florida clade 2 isolates. However, with the continued divergence of the two clades, primer sequences may need to be modified to remain optimal and should be compared against recent equine influenza virus sequences available on public sequence databases. The HA gene is amplified as a set of four overlapping PCR products, whilst three overlapping amplicons are produced from the NA gene. Each amplicon is between 400 and 600 bp in length. Equine influenza H3N8-specific PCR primers are tagged at the 5' end with M13 forward or reverse primer sequences, simplifying subsequent sequencing reactions. The protocol below describes a general method and may need adapting for use with different enzymes and commercial kits.

~~1.5.2.~~ 1.6.1. cDNA synthesis

cDNA is transcribed from viral RNA (extracted as described above) by reverse transcription (RT). Reverse transcriptase enzymes should be used following the manufacturer's

instructions. First, 4 µl RNA is denatured in the presence of a modified UNI-12 primer (5'-AGT-AGC-RAA-AGC-AGG-3', final concentration 1 µM) and 7 µl water at 70°C for 10 minutes before cooling on ice. Following this, dNTP mix (final concentration 0.5 µM each dNTP), DTT (10 mM final concentration), 1 × reaction buffer and 200 U reverse transcriptase enzyme are added to a final reaction volume of 20 µl. The reaction is incubated at 42°C for 1 hour followed by an inactivation step at 65°C for 20 minutes. A negative RT control should also be included where water is substituted for the test RNA.

4.5.3. 1.6.2. PCR amplification of HA and NA gene segments.

A high-fidelity DNA polymerase enzyme is used to amplify the HA and NA gene segments by PCR. For each amplicon, 50 µl PCR reactions are made consisting of 2 µl cDNA, dNTP mix (0.2 µM each dNTP), 1 × reaction buffer, 1U DNA polymerase, water and primer pairs (final concentration each of 0.2 µM) as listed in Table 2. The following cycling conditions may need adjusting depending on the DNA polymerase used: initial denaturation at 98°C for 30 seconds, followed by 30 cycles of 98°C for 10 seconds, 50°C for 30 seconds and 72°C for 30 seconds and a final extension at 72°C for 2 minutes. A negative control should be included for each primer pair where the product from the negative RT control is substituted for the cDNA.

Table 2. ~~Primer sequences for amplifying the HA and NA gene segments of H3N8 equine influenza virus by PCR for sequencing~~

Primer name	Primer sequence (5'–3')	Approximate nucleotide coverage
HA/AF	GC-GTAAAACGACGGCCAGT AGCAAAAGCAGGGGACGATATT	1–515
HA/AR	GC-AACAGCTATGACCATG GATTTGTTAGCCAATTCAG	
HA/BF	GC-GTAAAACGACGGCCAGT CAGGTGTCACTCAAAACG	428–1032
HA/BR	GC-AACAGCTATGACCATG GGATTTGCTTTTCTGGTAC	
HA/CF	GC-GTAAAACGACGGCCAGT GGTACATATGGAAATGCC	939–1336
HA/CR	GC-AACAGCTATGACCATG GAGCCACCAGCAATTCT	
HA/DF	GC-GTAAAACGACGGCCAGT GAAGGAAGAATTCAGGA	1251–1733
HA/DR	GC-AACAGCTATGACCATG GAGTAGAAACAAGGGTGTTTTAAAG	
NA/AF	GC-GTAAAACGACGGCCAGT AGCAAAAGCAGGAGTTT	1–508
NA/AR	GC-AACAGCTATGACCATG GCCCTATTTTGACACTC	
NA/BF	GC-GTAAAACGACGGCCAGT CACACAGGGCTCATTAG	417–1049
NA/BR	GC-AACAGCTATGACCATG CCGAAACCTTTTACACCG	
NA/CF	GC-GTAAAACGACGGCCAGT CACAGTTGGATATTTGTG	951–1461
NA/CR	GC-AACAGCTATGACCATG AGTAGAAACAAGGAGTT	

Table 3. *Primer sequences for amplifying the HA and NA gene segments of H3N8 equine influenza virus by PCR for sequencing*

Pathogen/ target gene	Primer (5'–3')	Concentration	Cycling parameters ^(a)
Method 1: Rash <i>et al.</i> , 2014; amplicon sizes for HA-A, B, C and D segments are 515bp, 605bp, 398bp and 483bp, respectively; amplicon sizes for NA-A, B and C segments are 508bp, 633bp and 511bp, respectively]			
HA	HA/AF: GC-(b) <u>GTAAAACGACGGCCAGT</u> -AGC-GAA-AGC-AGG-GGA-CGA-TAT-T HA/AR: GC-(c) <u>AACAGCTATGACCATG</u> -GAT-TTG-TTA-GCC-AAT-TCA-G	200 nm 200 nm	30 cycles of: 98°C/10 sec, 50°C/30 sec and 72°C/30 sec, and final extension 72°C/2 min
	HA/BF: GC- <u>GTAAAACGACGGCCAGT</u> -CAG-GTG-TCA-CTC-AAA-ACG HA/BR: GC- <u>AACAGCTATGACCATG</u> -GGA-TTT-GCT-TTT-CTG-GTA-C		
	HA/CF: GC- <u>GTAAAACGACGGCCAGT</u> -GGT-TAC-ATA-TGG-AAA-ATG-CC HA/CR: GC- <u>AACAGCTATGACCATG</u> -GAG-CCA-CCA-GCA-ATT-CT		
	HA/DF: GC- <u>GTAAAACGACGGCCAGT</u> -GAA-GGA-AGA-ATT-CAG-GA HA/DR: GC- <u>AACAGCTATGACCATG</u> -GAG-TAG-AAA-CAA-GGG-TGT-TTT-TAA-C		

NA	NA/AF: GC- <u>GTAAAACGACGGCCAGT</u> -AGC-AAA-AGC-AGG-AGT-TT NA/AR: GC- <u>AACAGCTATGACCATG</u> -GCC-CTA-TTT-TGA-CAC-TC		
	NA/BF: GC- <u>GTAAAACGACGGCCAGT</u> -CAC-ACA-GGG-CTC-ATT-AC NA/BR: GC- <u>AACAGCTATGACCATG</u> -CCG-AAA-CCT-TTT-ACA-CCG		
	NA/CF: GC- <u>GTAAAACGACGGCCAGT</u> -CAC-AGT-TGG-ATA-TTT-GTG NA/CR: GC- <u>AACAGCTATGACCATG</u> -AGT-AGA-AAC-AAG-GAG-TT		

^(a)A denaturation step prior to cycling has not been included see above.

^(b, c)PCR primers are tagged with M13 forward^(b) or M13 reverse^(c) sequences shown underlined in bold.

A convenient one-step method combining cDNA synthesis and PCR amplification has also been described by Gahan *et al.* (2019) using the primers above or alternative HA and NA primer sequences by Gildea *et al.* (2018).

The PCR products can be visualised following electrophoresis on a 0.8% agarose gel stained with a nucleic acid stain. PCR products may be purified using a commercial kit or by ethanol precipitation. Sequencing reactions are carried out using chain-terminating dideoxynucleotides and the M13 forward (5'-GTA-AAA-CGA-CGG-CCA-GT-3') and reverse (5'-AAC-AGC-TAT-GAC-CATG-3') primers for each PCR product to determine the sequence on both DNA strands. The final sequences can be assembled from the overlapping amplicons and any primer-derived sequence can then be removed.

Whole genome sequences can be rapidly determined using next generation sequencing technology, which uses universal primers and is rapidly becoming more accessible (Wasik *et al.*, 2023).

~~Although the genetic sequence of isolates can also be derived from PCR assays it remains essential to isolate infectious virus in order to examine the antigenic properties of new isolates and evaluate antigenic drift in the field.~~

1.7. Point-of-care tests for agent identification and characterisation

Point-of-care tests for the detection of H3N8 equine influenza virus include a reverse-transcription loop-mediated isothermal amplification assay (RT-LAMP) (Nemoto *et al.*, 2011) and an insulated isothermal RT-PCR (iiRT-PCR) assay using the portable POCKIT™ Nucleic Acid Analyzer (Balasuriya *et al.*, 2014). Both tests appear to provide rapid detection, high sensitivity and ease of use but require further validation in the field. Long-read sequencing technology using indirect sequencing also appears promising for virus characterisation outside the laboratory setting (Kleij *et al.*, 2024).

2. Serological tests

Influenza infections can be detected by performing serological tests on paired sera to show a rise in specific antibody. ~~These tests should be carried out whether virus isolation has been attempted or not. They~~ These tests are robust and may prove positive in the absence of virus ~~isolation detection~~. Two simple methods exist, HI and single radial haemolysis (SRH), each equally efficient and widely used. ELISAs for antibodies against influenza nucleoprotein are available but while less commonly used but have been shown to be robust and are useful for testing large numbers of samples (Galvin *et al.*, 2013, Kittelberger *et al.*, 2011, ~~Sergeant *et al.*, 2014~~). The complement fixation (CF) test can also be applied, but is not in general use. The paired serum samples, i.e. the acute and convalescent samples, should be tested together to minimise the impact of inter-assay variability. ~~The standard antigens are described above (Section B.1.3). Antigens are available from WOA Reference Laboratories.~~ If available, isolates from recent cases should be included. Freeze-dried post-infection equine antisera to A/eq/Newmarket/77 (H7N7), A/eq/Newmarket/1/93 (American lineage H3N8), A/eq/Newmarket/2/93 (European lineage H3N8), A/eq/South Africa/4/03 (Florida Clade 1, sublineage of American lineage), A/eq/Richmond/07 (Florida Clade 2, sublineage of American lineage) ~~and an influenza negative equine serum,~~ are available from the European Directorate for the Quality of Medicines (EDQM)². These sera have been assigned SRH values through ~~an international collaborative study studies~~ and can be used as primary reference sera for the assay (Daly *et al.*, 2007; ~~Mumford, 2000; Paillot *et al.*, 2020~~).

² Headquarters: EDQM – Council of Europe, 7 allée Kastner, CS 30026, F-67081 Strasbourg, France.

2.1. Haemagglutination inhibition test

~~The H3N8~~ antigen is first treated with Tween 80/ether to increase the sensitivity of the test (John & Fulginiti, 1966) ~~particularly for H3N8 viruses~~. The HI test can also be performed without ether treatment although at reduced sensitivity for H3N8. If included the H7N7 antigen is used untreated. The test is best done in V-bottomed microtitre plates using the appropriate dilution equipment. The test should be processed in batches, with a maximum of four plates of each antigen per batch. A HA row must be included on the first plate of each batch and each plate within the batch must include an EDQM positive reference serum. A negative control must also be included once per batch of samples being tested. The determination of 4 HA units should be carried out prior to commencement of the HI test. A macrotest may be used, for which antigen is diluted to a final HA titre of 1/8 per well and the volumes for PBS, sera and antigen are 0.5 ml. Sera are pretreated to remove nonspecific haemagglutinins, then inactivated at 56°C for 30 minutes. Pretreatments include the use of one of the following: (a) kaolin and RBCs absorption, not recommended for H7N7 HI, (b) potassium periodate (c) trypsin heat-treated potassium periodate-packed RBC treatment, or (e-d) Vibrio cholerae receptor-destroying enzyme. Potassium periodate ~~or V. cholerae receptor destroying enzyme~~ is the treatment of choice. The treated sera are diluted in PBS or PBS with 0.05% BSA, a standard dose of antigen is added (HA titre of 1/4 per well for microtitration assay), and these are kept incubated at room temperature ($23^{\circ}\text{C} \pm 2-15-24^{\circ}\text{C}$) for 30 minutes. After gentle mixing, RBCs are added and the test is read 30-45 minutes later. ~~The HI titres are read as the highest dilution of serum giving complete inhibition of agglutination. Either Chicken RBCs (0.5% or 1% [v/v] packed cells) in V-bottomed microtitre plates or guinea pig RBCs (0.5% [v/v] packed cells) in V or U-bottomed plates may be are used. If chicken RBCs are used, The plates may are read by tilting to 70° so that non-agglutinated cells 'stream' to the bottom of the well; the absence of this 'tear-drop' is evidence of partial agglutination. Non-agglutinated guinea pig cells appear as a 'button' in the bottom of the well and may take longer to settle.~~ The HI titre is the reciprocal of the greatest dilution showing complete inhibition of agglutination. Currently, a cut-off point for positive samples has not been determined for the HI test and thus, low titres should be investigated further. Titre increases of fourfold or more between paired sera indicate recent infection.

2.1.1. Tween 80/ether treatment of the virus

- i) To 39.5 ml of infective allantoic fluid, add 0.5 ml of a 10% (v/v) suspension of Tween 80 in PBS to give a 0.125% (v/v) concentration of Tween 80.
- ii) After mixing gently at room temperature for 5 minutes, add 20 ml of diethyl ether to give a final concentration of 33.3% by volume, and mix the suspension well at 4°C for 15 minutes.
- iii) After allowing the layers to separate by standing, remove the aqueous layer containing the disrupted virus particles to a glass bottle with a loose lid and allow the excess ether to evaporate off overnight (John & Fulginiti, 1966). Safety precautions while handling ether must be strictly observed and work should be confined to a fume hood.
- iv) Store treated virus in aliquots at -70°C.

2.1.2. ~~Titration~~ Determination of haemagglutination titre of the virus antigen

- i) Add 25 µl of PBS or PBS with 0.05% BSA solution to ~~all wells~~ 2 to 12 in a each row of a V-bottomed microtitre plate. Note: 0.05% BSA solution (0.5 ml of 30% BSA in 0.85% NaCl solution/300 ml PBS).
- ii) Add ~~25-50~~ µl of virus to first well (neat) then generate a twofold dilution series across the plate leaving the last well as a negative control.
- iii) Add an extra 25 µl of PBS ~~to all wells or PBS with 0.05% BSA solution to all wells.~~ Incubate at room temperature (15°C-24°C) for 30 minutes (± 2 minutes).
- iv) Add 50 µl of 0.5 or 1% chicken RBCs to all wells. ~~Leave~~ Incubate at room temperature ~~or at 4°C (particularly if ambient temperatures are high), (15°C-24°C) for 30-45 minutes (± 2 minutes).~~
- v) Read the results by tilting the plate to 70° so that non-agglutinated cells 'stream' to the bottom of the well. The HA titre (equivalent to 1 HA unit) is taken as the last virus highest dilution giving partial HA of antigen capable of causing agglutination.

2.1.3. Potassium periodate pretreatment of sera

- i) Freshly prepare 0.016 M potassium periodate (KIO₄) (0.1905 g in 50 ml PBS) solution. Heat at 56°C (± 1°C) until completely dissolved for at least 2 hours

- ii) Mix one volume (150 µl) of serum with two volumes (300 µl) of the freshly prepared 0.016 M KIO₄ potassium periodate (0.38 g in 100 ml PBS) solution, and leave incubate at room temperature (15°C–24°C) 22°C (± 2°C) for 15 minutes.
- iii) Add a further one volume (150 µl) of 3% glycerol in PBS to neutralise any excess periodate solution. Mix and leave incubate at room temperature (23°C ± 2°C) (15°–24°C) for 15 minutes.
- iv) Inactivate in a 56°C water bath for 30 minutes.

2.1.4. Test procedure

- i) Dispense 25 µl of PBS or PBS with 0.05% BSA solution to all wells of the V-bottomed microtitre plate except for the first well of the HA row.
- ii) Add serum (25 µl) to the first well of a row of 12, then generate twofold serial dilutions (1/8 to at least 1/512, allowing for dilution of 1/4 from treatment of serum), leaving the last well as a serum control. Add 25ml of PBS or PBS with 0.05% BSA solution to the serum control wells.
- iii) Dilute the antigen to give a dose of 4 HA units (4 × minimum agglutinating dose, i.e. titre 1 HA unit/4).
- iv) Add 25 µl of the antigen to each all wells of the test plate except the serum control wells and the wells of the HA control row. To the HA control row, add 50ml of the appropriate antigen to the first well and double dilute 25ml across the row. Add an additional 25ml of PBS or PBS with 0.05% BSA to all wells of the HA row and the serum control wells. and Incubate at room temperature (15°C–24°C) for 30 minutes.
- v) Add 50 µl of 0.5 or 1% chicken RBCs to each well. Leave at room temperature or at 4°C (particularly if ambient temperatures are high), for 30 minutes. Incubate at room temperature for 45 minutes.
- vi) The plates may be read by tilting to 70° so that non-agglutinated cells 'stream' to the bottom of the well. Absence of this 'tear-drop' is evidence of partial agglutination. No agglutination is recorded as a positive result.

2.2. Single radial haemolysis

In this test, viral antigens are coupled to fixed RBCs that are suspended in agarose containing guinea-pig complement (C'). Wells are punched in the agarose and filled with test sera. Influenza antibodies and C' lyse the antigen-coated RBCs, resulting in a clear, haemolytic zone around the well; the size of this zone is directly proportional to the level of strain-specific antibody in the serum sample (Cullinane *et al.*, 2020; Entenfellner *et al.*, 2020; Morley *et al.*, 1995). The test is carried out over 3 days.

Simple immunodiffusion plates (MP Biomedical) may be used for the assay, but simple Petri dishes are also suitable used for the assay, but special immunodiffusion plates (MP Biomedical) are also available. Sheep RBCs collected into Alsever's solution are washed three times. The C' can be obtained commercially, or normal guinea-pig serum can be used. The viral antigens are egg-grown stocks or purified preparations; the strains used are the same as for the HI tests. The viruses are coupled to RBCs by potassium periodate or by chromic chloride. The coupled antigen/RBCs preparations are mixed with C', together with a 1% solution of agarose (low melting grade) in PBS. Optimisation of virus antigen, guinea-pig complement and chromic chloride should be carried out for each new batch of reagent to ensure clearest zones (non-hazy) are obtained.

Care must be taken to ensure that the temperature is not allowed to rise above 42°C at any time. The mixture is poured into plates and left to set. Wells of 3 mm in diameter and 42–20 mm apart are punched in the solidified agarose, at least 6–10 mm from the edge of the plates. Such plates may be stored at 4°C for 12 weeks–24 hours. Plates are prepared for each antigen.

Sera are inactivated at 56°C for 30 minutes, but no further treatment is necessary. Paired sera should be assayed on the same plate. As a minimum, a subtype-specific antiserum should be included as a control serum in one well on each plate. All sera are tested in a control plate containing all components except virus to check for nonspecific lysis. Alternatively, an unrelated virus, such as A/PR/8/34 (H1N1), may be used in the control plate. Sera that show haemolytic activity for sheep RBCs must be pre-absorbed with sheep RBCs. Zones of lysis should be clear and not hazy or translucent. All clear zones should be measured with digital calipers and the area of haemolysis calculated.

2.2.1. Preparation of reagents

- i) Saline/HEPES: 0.85% sodium chloride (NaCl) (~~4.25 g/500 ml~~ 17 g/2 litre); 0.05 M HEPES (N-2-hydroxyethylpiperazine, N-2-ethanesulphonic acid; ~~5.95 g/500 ml~~ 23.6 g/2 litre); and 0.02% sodium azide (0.4 g/2 litre). Make up to 2 litres with deionised water. Adjust to pH 6.5 with NaOH.
- ii) Saline/HEPES/0.05% BSA: as saline/HEPES with ~~0.2% (w/v)~~ 0.05% bovine serum albumin (BSA) solution (0.5 ml of 30% BSA in 0.85% NaCl solution/300 ml saline HEPES).
- iii) Saline/HEPES/0.2% BSA: as saline/HEPES with 0.2% BSA (1 ml of 30% BSA in 0.85% NaCl solution/150 ml saline HEPES).
- iv) CrCl₃ stock solution (2.25 M) 6 g/10 ml: Make fresh ~~1/400-350~~ dilution in 0.85% NaCl for each assay. Note: The dilution of CrCl₃ may need to be optimised in conjunction with the optimisation of viral antigen and guinea pig complement.
- v) PBS 'A': NaCl (~~10.00-20.00~~ g); KCl (~~0.25-0.5~~ g); Na₂HPO₄·2H₂O (~~1.45~~ 3.636 g); KH₂PO₄ (~~0.25-0.5~~ g); and Na azide (~~0.20-0.4~~ g). Make up to 4-2 litres with distilled-deionised water.
- vi) Agarose in PBS: ~~Place flask containing PBS 'A' on a stirrer. Slowly add 10 g agarose to the stirring solution. Liquefy in a pressure cooker. Dispense into glass bottles for storage at 22°C (± 2°C). Prepare a 1% agarose in PBS 'A' (1 g/100 ml) solution by slow addition of agarose. Adjust the volume as required allowing 13 ml per test plate. Autoclave for 15 minutes at 121°C (± 1°C) overnight (on pre-test day) without cooling. Incubate the agarose in a 56°C water bath to cool 1-2 hours prior to pouring the plates. Alternatively, the agarose solution can be liquefied in a microwave on the test day.~~
- vii) Virus antigen: Allantoic fluid containing infectious virus is harvested and stored at -70°C. Optimisation of virus antigen is carried out using a broad range of antigen dilutions with 0.2 ml difference in antigen volume e.g. 0.6 ml, 0.8 ml, 1.0 ml, 1.2 ml, 1.4 ml, 1.6 ml, 1.8 ml, 2.0 ml added to 3 ml 8% RBCs. Note the total volume of blood and virus equates to half the volume of CrCl₃. The expected pattern of results will be smaller clearer zones of lysis as the volume of virus antigen increases. Optimum volume of virus antigen is that which results in the largest and clearest zones with appropriate reference serum. A short titration curve determines the optimum ratio of virus antigen to RBCs to be used when preparing sensitised sheep RBCs. The H7N7 influenza strains always produce clear zones; the H3N8 strains sometimes produce. If the zones are hazy zones, in which case it is may be necessary to concentrate the virus by centrifugation.
- viii) Sheep blood: Collect blood into an equal volume of Alsever's solution and store at 4°C. It may be necessary to test bleed several sheep, as characteristics of RBCs from individual sheep vary. ~~Keep The blood for 2 days before use, it may then be usable for up to 3-2 weeks, providing sterility is maintained.~~
- ix) Complement: Use commercially available guinea-pig complement or collect serum from young guinea-pigs of 300-350 g body weight and store in small volumes at -70°C. For use, thaw in cold water and hold at 4°C prior to mixing. The optimum volume of guinea-pig complement should be determined for each new batch by evaluating a range of volumes e.g. 0.25 ml, 0.5 ml, 0.75 ml.
- x) Treatment of sera: Use undiluted sera heat inactivated at 56°C for 30 minutes. Avoid repeated freeze-thaw cycles. Equine anti-sera provided by the EDQM are not heat inactivated before use.

2.2.2. Test procedure

Day 1 (pre-test day):

- i) Prepare the saline/HEPES, saline/HEPES/0.05% BSA, PBS 'A' and 1% agarose as above. Wash sheep RBCs at least three times in saline/HEPES/0.05% BSA solution by centrifugation at 935 g for 10 minutes.
- ii) Prepare an appropriate volume of 8% RBCs (v/v packed cells) in saline/HEPES/0.05% BSA solution, having first calculated the number of plates required and allowing 4-3 ml of 8% RBCs per Petri dish (6 × 11.92 × 16 mm) cm immunoplate and 4-2-10 ml extra.

Day 2 (test day)

- i) Prepare the saline/HEPES/0.2% BSA and the CrCl₃ (1/350 dilution of 2.25 M CrCl₃ stock in 0.85% NaCl) solutions.

- ii) Pre-warm universals in a 42°C waterbath. Pre-warm Petri dishes by incubation at 34°C.
- iii) Aliquot 3mls of 8% RBCs per plate in a universal. Add a predetermined volume³ of virus antigen to the 8% RBCs solution. (Note: For negative control plates add PBS 'A' or negative allantoic fluid in lieu of virus). ~~Hold~~ Incubate the mixture at 4°C room temperature 15–24°C for 10 minutes. ~~Haemagglutination may be observed.~~
- iv) SLOWLY add CrCl₃ (1/400–350 in 0.85% NaCl) at half the total volume of virus/RBCs suspension. ~~Hold~~ Incubate at 22°C (±2°C) room temperature 15–24°C for 5 minutes with occasional mixing rotation.
- v) Sediment the sensitised RBCs by centrifugation at 4500–526 g for 5 minutes.
- vi) Gently resuspend in approximately 10 ml saline/HEPES/0.02% BSA and centrifuge at 4500–526 g for 5 minutes.
- vii) Resuspend RBCs to an 8% suspension in PBS 'A'.
- viii) During the sensitisation process, melt the agarose if necessary or transfer premade agarose from 56°C to 42°C. Shortly before use, pipette 7.8–13 ml volumes to pre-warmed Universal bottles and retain at 42°C. ~~Check that the agar has cooled to 42°C before use.~~

2.2.3. Preparation of plates

- i) Add 0.9–1.5 ml of virus sensitised sheep RBCs (virus-sensitised/unsensitised for control) to 7.8–13 ml agarose (42°C). Mix quickly, but gently.
- ii) Add 0.3 ml the determined optimum volume⁴ of undiluted guinea-pig serum. Mix again very gently by inversion/ rotation and pour into immunoplates pre-labelled, pre-warmed 9 cm Petri dishes on a levelling table. Allow to set and air dry without a lid for 5 minutes.
- iii) Place lids on plates and store the plates inverted at 4–2–8°C in a humid box until used until ready to use.
- iv) Prepare control plates with unsensitised cells or cells sensitised with an unrelated virus. Batches of prepared plates can be stored for several weeks.
- iv) Punch 3 mm holes in the set gels to a prepared template, allow for 46–8 test sera, and a positive control serum and a negative control serum per plate. On antigen control plates, prepare five rows of eight wells.
- vi) Remove any excess liquid from the test wells if necessary. Pipette 10 µl of heat-inactivated (56°C for 30 minutes) test sera and a positive control serum sera to appropriate wells. Incubate at 34°C for 20 hours in a humid box overnight in a humid chamber.

Day 3

- vi) Read plates when all experimental controls are within range (~16–22 hours).
- vii) Measure zone diameters. Two diameter readings of each zone of lysis are taken using digital calipers. The plate is rotated approximately 180 degrees between each diameter read. Zones of haemolysis are generally circular but if not precisely circular, it is recommended to rotate the plate at 90 degrees and take the average. One diameter reading of <3 is recorded for samples that read negative, and-. Calculate areas of haemolysis in mm² after the area of the well has been deducted.

2.2.3.4. Interpretation of the results

For results to be valid, positive and negative control sera should give results assigned through the international collaborative study (Daly *et al.*, 2007; Mumford, 2000; Paillot *et al.*, 2020) or if not using these international standards, results consistent with those expected on the basis of prior experience. Areas of haemolysis for the control sera should be clear and intra-laboratory variation should be no more than 5% for the control serum. ~~Results may be expressed as mm² or as a ratio of the control serum value.~~ Sera giving positive results in the control plate should be adsorbed with sheep RBCs. For diagnostic purposes, acute and convalescent sera should be tested in duplicate on the same plate. Increase in zone areas produced by convalescent serum compared with acute serum is evidence of infection. An increase in area of 25 mm² or 50% whichever is smaller, is routinely deemed to be significant

3 Determined by optimisation of viral antigen. Prepare three plates by adding 0.6, 1.2 or 1.8 ml of virus antigen to 2 ml RBCs. Add 1.3, 1.6 and 1.9 ml CrCl₃ respectively and resuspend to 2 ml in PBS 'A'. Optimum volume of virus antigen is that which results in the largest and clearest zones with appropriate reference serum.

4 Determined by optimisation of each new batch of guinea-pig complement (see Section B.2.2.1 step ix).

~~but this depends on the reproducibility of the test within the laboratory, and should be equivalent to a twofold or more increase in antibody concentration. This area can be calculated from a standard curve generated from a dilution series of a standard antiserum.~~

2.3. Competitive/blocking ELISA

Several competitive/blocking ELISAs have been described, based on influenza A virus nucleoprotein and a monoclonal anti-nucleoprotein horseradish peroxidase conjugated antibody. Four readily available ELISAs, originally developed for avian influenza, have been evaluated and shown to be effective in the detection of influenza A antibodies in horses (Kittelberger *et al.*, 2011). The specific method supplied with each assay should be followed. Galvin *et al.* (2013) found that a multispecies ELISA for the detection of antibodies to the internal nucleocapsid of the influenza A virus in bird, pig and horse sera was less sensitive than either the HI or SRH test for the diagnosis of equine influenza.

C. REQUIREMENTS FOR VACCINES

1. Background

1.1. Rationale and intended use of the product

Equine influenza is usually a self-limiting disease and its economic significance is primarily due to the contagious nature of the virus and the disruption to equestrian activities. Outbreaks in naïve populations can result in fatalities (Diallo *et al.*, 2021). Rapid diagnosis, movement restrictions and vaccination are the key control measures for equine influenza. Vaccination decreases clinical signs and virus shedding. Influenza vaccines are widely available and are routinely used in competition horses in Europe, the Americas, and Asia. In some countries, vaccination is mandatory for sport horses and racehorses that are competing under rules of equestrian organisations. Following a primary course of three doses at intervals of around 0, 1 and 6 months, an annual booster is the usual minimum requirement. More frequent vaccination is recommended for young horses and some equestrian organisations require biannual boosters.

Equine influenza virus vaccines are typically whole consist of inactivated, subunit or canary pox-vectored vaccines ~~whole viruses or their glycoprotein subunits, with or without adjuvant. Live attenuated virus vaccine and canary pox vectored vaccines have~~ become available commercially in some countries. Immunity generated by inactivated vaccines administered via the intramuscular route is reliant on stimulation of circulating antibody to the HA, which neutralises virus; some products have been shown to stimulate antibody in respiratory secretions. Critically the integrity and conformation of the HA should be maintained during inactivation procedures to ensure that the vaccine stimulates appropriate neutralising antibody. This can be tested by use of an immunological assay such as SRD (single radial diffusion), which measures immunologically active HA capable of reacting with specific anti-HA antibodies. The immunogenicity of the vaccine can be confirmed by measurement of HA antibody stimulated in small animal models or the target species. Several equine influenza vaccines including inactivated whole virus, subunit and a canary pox-vectored vaccine have also been shown to have the potential for cell-mediated immunity (CMI) priming (Gildea *et al.*, 2013b).

Live virus-vectored vaccines, which contain the influenza virus gene encoding HA rather than antigen, cannot be potency tested by SRD. Instead, the infectious titre of the recombinant is used as an *in-vitro* measure of potency and immunogenicity is assessed by measurement of antibody stimulated in the target species.

Antibody to HA as measured by SRH, stimulated by inactivated whole virus, subunit or canary pox-vectored vaccine correlates well with protection against infection in an experimental challenge model system (Edlund Toulemonde *et al.*, 2005; Mumford *et al.*, 1983; 1994). In contrast, a cold-adapted temperature-sensitive mutant used as a live attenuated vaccine replicates in the upper respiratory tract and does not stimulate high levels of circulating antibody to HA but nevertheless provides protection against challenge infection (Whitaker-Dowling *et al.*, 2001). Immunity is presumed to be mediated through mucosal or cellular responses rather than circulating antibody. As with the vectored vaccine, in-process control testing is reliant on measurement of the infectious virus titre.

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 as manufacturers are obliged to meet European Pharmacopeia, USDA or other national and regional requirements.

All equine influenza vaccines should contain epidemiologically relevant strains

A formal equine influenza global surveillance programme has been in place since 1995. The WOAHP Reference Laboratories and other collaborating laboratories collect data on outbreaks of equine influenza and strain variation that is reviewed annually by an Expert Surveillance Panel (ESP) including representatives from WOAHP and WHO. This panel makes recommendations on the need to update vaccines, and these are published annually in the *WOAH Bulletin* and on the WOAHP website. The criteria for updating equine influenza vaccines are similar to those for human influenza vaccines and based on evidence of vaccination breakdown in the field and analysis of antigenic changes, supported by genetic changes and, when possible, supporting experimental challenge data.

~~H7N7: Many vaccines still contain an H7N7 strain. However, The Expert Surveillance Panel has recommended that the H7N7 component should be omitted as reports of infections with this subtype have not been substantiated during the past 30 years it is not necessary to include an H7N7 virus in vaccines as these viruses have not been detected over the course of many years of surveillance and are therefore presumed not to be circulating.~~

H3N8: Antigenic and genetic variants of H3N8 viruses co-circulate (Bryant *et al.*, 2009; Favaro *et al.*, 2018) and it is important to include a strain or strains that are epidemiologically relevant as recommended by the WOAHP Expert Surveillance Panel. WOAHP Reference Laboratories can provide help in selecting suitable vaccine strains.

2. Outline of production and minimum requirements for conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

For each vaccine strain, a prototype batch should be prepared to establish its suitability as a vaccine strain, i.e. purity and safety should be confirmed by standard techniques. The ability of seed-lot viruses to grow to high titre and generate sufficient antigenic mass to stimulate adequate antibody responses in the target species, should be confirmed. ~~Additionally, Vaccine virus derived in MDCK cells should be fully characterised to ensure that antigenic variants have not arisen during the culture process, such that the vaccine virus is no longer representative of the original isolate.~~

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

Virus strains may be obtained from WOAHP Reference Laboratories⁵. Viruses selected as vaccine strains should be described in terms of origin and passage history. The strains are propagated in the allantoic cavity of 10-day-old embryonated hens' eggs or cell cultures, such as MDCK. All manipulations must be conducted separately for each strain. Viral growth is monitored by HA tests. Passaged virus is identified by serological tests, such as HI or SRH₂, or by PCR using specific primers. If vaccine virus is grown in cell culture, antigenic studies with ferret sera and monoclonal antibodies (MAbs) should be undertaken to ensure that variant viruses have not been selected during passage to prepare master and working seed viruses. Master and working seed viruses are divided into aliquots and stored in freeze-dried form at -20°C or at -70°C following testing for extraneous agents. Records of storage conditions should be maintained.

The master seed lot of each vaccine strain selected should be processed at one time to assure a uniform composition, tested for extraneous agents, and fully characterised. Antisera or MAbs for use in HI tests to characterise vaccine strains may be obtained from WOAHP and WHO Reference Laboratories.

Working seed lots are derived from a master seed lot and should be of uniform composition, free from extraneous agents, and fully characterised. Aliquots of the working seed are used for production of vaccine.

Master and working seed lots should be prepared in specific pathogen free eggs or, as a minimum, in eggs derived from a healthy flock.

5 <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>

If MDCK cells are used to propagate vaccine virus, master cell lines should be established and stored in liquid nitrogen, and should be tested for freedom from extraneous agents according to National Control Authority Guidelines.

Examination of seed viruses for extraneous agents including mycoplasmas and other equine viruses should be performed by appropriate techniques, including inoculation of susceptible tissue cultures and examination for cytopathic effect or application of fluorescent antibodies for antigen detection.

The presence of other common equine respiratory pathogens, e.g. equine herpesviruses 1, 2, 4, equine picornaviruses, equine viral arteritis, and equine adenoviruses, should be specifically excluded.

The absence of bacteria should be confirmed by standard sterility tests and toxicity tests in small animals.

2.2. Method of manufacture

2.2.1. Procedure

Production is based on a seed-lot system that has been validated with respect to the characteristics of the vaccine strains. Where eggs are used, each strain of virus is inoculated separately into the allantoic cavity of 9–11-day-old embryonated hens' eggs from a healthy flock. The eggs are incubated at a suitable temperature for 2–3 days, and the allantoic fluid is collected. Alternatively, each strain is inoculated separately into MDCK cell cultures. The viral suspensions of each strain are collected separately and inactivated. If necessary, they may be purified. Suitable adjuvants and antimicrobial preservatives may be added.

Monovalent virus pools should be inactivated as soon as possible after their preparation, by a method approved by the National Control Authority. If formalin (37% formaldehyde) or beta-propiolactone (2-oxetanone) is used, the concentration by volume should not exceed 0.2%. Ideally, pools should be held at 4°C and should be inactivated within 5 days of harvest. Inactivation of the vaccine must be demonstrated. A suitable method consists of inoculating 0.2 ml of undiluted monovalent pool and 1/10 and 1/100 dilutions of the monovalent pool into the allantoic cavities of groups of fertile eggs (10 eggs in each group), and incubating the eggs at ~~33–37~~³⁴°C for 3 days. At least 8 of the 10 eggs should survive at each dosage level. A volume of 0.5 ml of allantoic fluid is harvested from each surviving egg. The fluid harvested from each group is pooled, and 0.2 ml of each of the three pools is inoculated, undiluted, into a further group of 10 fertile eggs. Haemagglutinin activity should not be detected in these new groups of eggs. In some countries, the requirement that 80% of the eggs should survive during incubation may be impossible to satisfy, in which case the National Control Authority should then specify a modified requirement to be satisfied. Before inactivation, samples should be collected for bacterial and fungal sterility tests.

Monovalent material may be concentrated and purified by high-speed centrifugation or other suitable methods approved by the National Control Authority, either before or after the inactivation procedure. It is important to concentrate and purify the virus under optimum conditions, e.g. temperatures that preserve its antigenic properties.

The monovalent virus pool shall be shown not to contain viable influenza virus when tested by inoculation of embryonated hens' eggs, by a method approved by the National Control Authority. Alternatively, the satisfactory inactivation can also be demonstrated by inoculating monolayers of MDCK cells.

2.2.2. Requirements for substrates and media

Vaccines should be produced in eggs or in a cell line that meets the requirements of the National Control Authority. Wherever practicable the use of substances of animal origin for example, serum and trypsin should be kept to a minimum. Substances of animal origin used during production should be subjected to a suitable, validated sterilisation or inactivation procedure or be tested for the absence of extraneous organisms.

2.2.3. In-process controls

Relevant in-process controls should be applied before and after inactivation and before and after concentration and purification.

In-process controls include: (a) identity of virus strains (tested by HI); (b) sterility; (c) virus titre; (d) haemagglutinin content (tested by chicken RBCs agglutinating units, CCA [chick cell agglutination]); and (e) immunologically active HA (tested by SRD or another suitable immunochemical method).

2.2.4. Single radial diffusion test

SRD is a reliable method for measuring immunologically active HA in terms of μg HA, and is used routinely for potency testing of human influenza vaccines (Wood *et al.*, 1983b).

The potency of inactivated equine influenza vaccine depends on the concentration of immunologically active haemagglutinin (Wood *et al.*, 1983a).

Assessment of the antigenic content of the vaccine by CCA alone may be misleading, as the sensitivity of this assay is a reflection of the ability of virus strains to agglutinate RBCs. Disruption of virus may lead to an apparent increase in HA as measured by CCA. The CCA assay does not provide a measure of the antigenic properties of the HA (HA may retain its properties to bind to RBCs while losing its ability to stimulate antibody).

Most equine influenza vaccines contain more than one variant of the H3N8 subtype. In this situation, it is not possible to judge the potency of individual H3N8 components from serological tests performed on sera collected from horses or small animals vaccinated with the final product, because of cross reactivity between the two isolates of the same subtype. Thus, it is important that a reliable method, such as SRD, be used to measure the potency of individual components before and after inactivation and prior to mixing and formulation with adjuvant.

In the SRD test, virus preparations are compared with a calibrated reference preparation of known HA content. Antigens are allowed to diffuse through a gel containing an antiserum specific for a particular HA. The distance diffused by the antigen before precipitation by the antibody incorporated in the gel is directly related to the concentration of haemagglutinin in the antigen preparations.

Standard reagents for SRD testing are available from the WHO International Laboratory for Biological Standards⁶. Reagents for the H3N8 strains A/eq/Miami/63, A/eq/Kentucky/81, A/eq/Newmarket/1/93 (American lineage) and A/eq/Newmarket/2/93 (European lineage) are currently available.

2.2.4. Final product batch tests

i) Sterility and purity

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

ii) Safety

- a) For inactivated or subunit vaccines absence of viral infectivity should be demonstrated by two passages in 10 embryonated hen eggs. The allantoic fluid should have no haemagglutinating activity.
- b) Using no fewer than three horses, each horse is inoculated intramuscularly (at two different sites) with the dose of vaccine specified by the manufacturer; these inoculations are repeated 2–4 weeks later. The animals are kept under observation for 10 days after the second set of injections. No abnormal local or systemic reaction should ensue.
- c) If vaccine is to be used in mares, safety should be demonstrated by giving two doses of vaccine to no fewer than two pregnant mares at the prescribed interval within the trimester for which the vaccine is recommended. Once safety has been demonstrated on a prototype batch, safety testing in pregnant mares may be omitted for routine testing of subsequent batches of the final product.

iii) Batch potency

Following mixing of viral antigens and adjuvants, aliquots should be potency tested *in vivo* using horses and laboratory animals (guinea-pigs or mice) or a suitable alternative immunochemical assay. Adjuvants cause interference in quantitative *in-vitro* tests, such

⁶ National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, UK.

as CCA and SRD, although SRD may be used on the final product as a qualitative assay to demonstrate the presence of antigen for each vaccine strain. For repeated batch tests, only guinea-pig laboratory animals or preferably a suitable alternative immunochemical assay are used, subject to agreement of the National Control Authority.

a) Serological responses in horses

For a valid *in-vivo* potency test, naive seronegative horses must be selected for vaccination. Young horses or ponies (not less than 6 months old) should be screened for the presence of antibody using ~~H7N7 and~~ H3N8 viruses including recently isolated viruses relevant to the area in which the horses were reared. If HI tests are used for screening, H3N8 viruses should be treated with Tween 80/ether to maximise the sensitivity of the test. Alternatively, SRH may be used to establish the seronegative status of animals.

To test a vaccine for efficacy in horses, inject a volume corresponding to one vaccine dose by the recommended route into each of five susceptible seronegative horses. After the period recommended between the first and second doses, as stated on the label, a volume of vaccine corresponding to the second dose of vaccine is injected into each horse. Unvaccinated horses may be co-pastured with the vaccinates as sentinels for wild type virus infection and bled on same schedule as vaccinates.

Three blood samples are collected from each animal, the first at the time of the first vaccination, the second 1 week after the first vaccination to check for anamnestic response, and the third 2 weeks after the second vaccination.

The serological assay used to measure the antibody response to the viruses contained in the vaccine must be standardised for a valid *in-vivo* potency test. The SRH assay (see Section B.2.2) is preferred as standard reference sera are available for quality control purposes from the European Pharmacopoeia⁷. These sera should be tested in parallel with the test sera to ensure that the test is valid with respect to sensitivity; the values obtained should not vary by more than 20% from the SRH values assigned in an international collaborative study (Daly *et al.*, 2007; ~~Mumford, 2009~~). Due to poor repeatability and reproducibility of the HI test between different laboratories, no HI titre could be assigned to these sera.

Following vaccination the antibody value measured by SRH should not be less than 150 mm². This is higher than the value required in the European Pharmacopoeia Monograph for inactivated equine influenza vaccines (85 mm²) as ~~this that~~ value is not considered to be protective. If the value found for any horse after the first vaccination indicates that there has been an anamnestic response, the result is not taken into account. A supplementary test is carried out, as described above, replacing the horses that showed an anamnestic response with an equal number of new animals. Similarly, if unvaccinated sentinels show elevation of titers during the test period, the entire experiment is suspect and must be repeated.

If the HI test is used, the antibody titre of each serum taken after the second vaccination in each test should not be less than 1/64 (calculated for the original serum, taking into account the predilution of 1/8). Alternatively, the antibody levels stimulated by the vaccine under test should be shown to be at least equal to the antibody levels stimulated by a standard vaccine tested in parallel that has been shown previously to protect horses against challenge infection.

b) Challenge studies in horses

Challenge studies may be carried out by exposing vaccinated horses/ponies to an aerosol of virulent influenza virus no fewer than 2 weeks and preferably more than 3 months after the second dose of vaccine. Comparisons of clinical signs, virus excretion and serological responses are made with a group of unvaccinated control animals challenged at the same time. The timing of the challenge procedure will reflect the claims to be made on the data sheet regarding duration of immunity. Protection at 2 weeks post-vaccination when antibody levels are at their peak level does not necessarily indicate good duration of immunity under field conditions.

If tests for potency in horses have been carried out with satisfactory results on a representative batch of vaccine, these tests may be omitted as a routine control on other

⁷ Serum to A/eq/Newmarket/1/77 (Catalogue number E0850010), A/eq/Newmarket/1/93 (E0850021), ~~and A/eq/Newmarket/2/93 (E0850022), and A/eq/South Africa/4/03 (Y0000712) and A/eq/Richmond/1/2007 (Y0001874).~~

batches of vaccine prepared using the same seed-lot system, subject to agreement by the National Control Authority.

c) Serological responses of guinea-pigs

Inject each of no fewer than five guinea-pigs free from specific antibodies with one vaccine dose. Collect blood samples 21 days later, and test the serum by SRH or HI (see Sections B.2.1 and B.2.2). Perform the tests of each serum using, respectively, the antigen(s) prepared from the strain(s) used in the production of the vaccine. The antibody titre of each serum in each test should not be less than the titre stimulated by a standard vaccine that has been shown to stimulate protective levels of antibody in horses.

2.3. Requirements for authorisation of vaccines

2.3.1. Safety requirements

The safety requirements may vary with the National Authority but usually include the assessment of the administration of an overdose and of the repeated administration of one dose to young horses and pregnant mares, if the vaccine is intended for use in pregnant mares. For live vaccines the dissemination by the vaccinated horse and the spread of the vaccine strain from vaccinated to unvaccinated horses along with the possible consequences must be investigated. Reversion to virulence studies should be performed by serial passaging of the live vaccine.

2.3.2. Efficacy requirements

The efficacy requirements may vary with the National Authority but usually include the assessment of the serological response in horses and virus challenge studies in susceptible horses (see Section C.2.2.5.iii.b).

Where claims for duration of immunity are made on the data sheet, these should be supported with data on the duration of protective levels of antibody maintained in horses vaccinated according to the recommended schedule. Antibody levels quoted as protective should be validated in challenge studies or by comparison with published reports.

2.3.3. Stability

Vaccines should be stored at $5\pm3^{\circ}\text{C}$ and protected from light. The shelf life quoted on the data sheet should be demonstrated by testing the potency of aliquots over time using the guinea-pig potency test (see Section C.2.2.5.iii.c).

2.4. Requirements for authorisation of strain updates to vaccines

To enable vaccine manufacturers to respond quickly to recommendations from the Expert Surveillance Panel, existing vaccines that have been updated in accordance with the WOA recommendations should be authorised if they meet the requirements for final product batch testing (see Section C.2.2.5).

The Committee for Veterinary Medicinal Products (CVMP) for the European Agency for the Evaluation of Medicinal Products (EMA) has developed Guidelines on the compliance of authorised equine influenza vaccines with WOA recommendations. In the USA, viral strain changes in equine influenza vaccines are addressed in the USDA/CVB/Veterinary Services Memorandum No. 800.111, which heed WOA recommendations.

3. Vaccines based on biotechnology

3.1. Vaccines available and their advantages

A canary pox recombinant vaccine is available in some countries and was used in the equine influenza control and eradication programme in Australia in 2007. A nucleoprotein ELISA was used to differentiate horses vaccinated with the recombinant vaccine from horses that had been exposed to virus by natural infection (DIVA). This DIVA was possible because the canary pox recombinant expresses only the haemagglutinin gene of equine influenza. ~~There is some evidence that this vaccine may be used to prime the immune system in the face of maternally derived antibodies (Minke et al., 2008).~~

3.2. Special requirements

National Authorities frequently require an environmental risk assessment. This may include an assessment of the potential direct and indirect, immediate or delayed adverse effects of the genetically modified vaccine on human and animal health and on the environment. The phenotypic and genetic stability of the vaccine and its potential interactions with other organisms may need to be evaluated.

4. Post-vaccination monitoring

Both the SRH and HI tests for the measurement of antibodies against the virus HA may be used for post-vaccination monitoring. However, the SRH test is internationally standardised and therefore considered the method of choice for post vaccination monitoring and evidence-based optimisation of vaccination regimes (Cullinane *et al.*, 2020). SRH antibody titres expressed as the area of haemolysis are correlated to protection. When there is no significant mismatch between the EIV vaccine viruses and circulating strains clinical signs of EI are significantly reduced when SRH antibody levels are $\geq 85 \text{ mm}^2$ and virus shedding is significantly reduced with SRH antibody levels of $\geq 154 \text{ mm}^2$.

In an endemic population where the HA canary pox recombinant vaccine is used, the nucleoprotein ELISA differentiates vaccinated horses from horses exposed to virus by natural infection (DIVA). This approach has been combined with SRH testing in the evaluation of vaccination coverage in France (Fougerolle *et al.*, 2019).

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NB: There are WOAHP Reference Laboratories for equine influenza (please consult the WOAHP Web site: <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3> <http://www.oie.int/>)
Please contact the WOAHP Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for equine influenza

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2019.

Appendix 1: Equine influenza
Intended purpose of test: population freedom from infection

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
Real-time RT-PCR ☒ ☒ Horse	NP swab; Matrix gene	Estimated using a two-test (nucleoprotein ELISA and RT-PCR) – in-two population BLCM DSe of RT-PCR was 99.3% (95%PI); DSp of RT-PCR was 99.8% (95%PI)	- A total of 1248 samples from three continents. The two populations were created based on country status for equine influenza; 276 horses in populations reported to be equine influenza free and 972 horses in countries where equine influenza was circulating ¹ - More than 32,000 tests applied to establishing population freedom in Australia ²	Validated by WOA Reference Laboratory in Ireland – report available on request	1. Very sensitive, detects both vaccinated and subclinical positive horses 2. Detects influenza type A strains including non-equine viruses that may jump the species barrier ³ 3. Well established internationally 4. High-throughput 5. Rapid 6. Proficiency panel of nasal samples available from Reference Laboratory	1. Highly sensitive –positive horses are not necessarily infectious 2. Possibility for contamination at sample collection or in laboratory leading to false positives 3. Need specialised equipment	¹ Gildea <i>et al.</i> (2018); ² Sergeant & Wilson (2011) ³ Heine <i>et al.</i> (2007)
RAD ☒ ☐ Horse	NP swab Influenza A viral nucleoprotein primarily for human viruses	Using RT-PCR as reference standard 22 RADs tested had DSe of 27% to 73% DSp was 100%	30 RT-PCR positive swabs from horses on eight premises where EI outbreaks were confirmed and 30 from horses in influenza free country	See reference	1. Rapid 2. Requires little technical expertise and no specialist equipment 3. Low risk of false positives	1. Risk of false negatives 2. Large differences in sensitivity of different RADs 3. Not high throughput	Yamanaka <i>et al.</i> (2016)
Antigen-capture ELISA ☒ ☐	NP swab Nucleoprotein	Using RT-PCR as reference standard DSe of ELISA was 35% DSp was 100%	- 75 swabs from 64 horses on seven premises where EI outbreaks were confirmed - Seven samples from seven seronegative healthy foals	See reference	1. Rapid 2. High specificity	1. Less sensitive than RT-PCR 2. Need specialised equipment 3. positive horses are not necessarily infectious	Galvin <i>et al.</i> (2014)
HI ☒ ☒ Horse	Serum Antibodies against equine influenza virus (EIV) haemagglutinin	N/A	391 horses of various ages in India ¹ 110 healthy horses on ten farms in Israel ²	N/A	1. Simplicity 2. Widely used 3. Cost-effective 4. Quicker than SRH	1. May not be useful if some horses are vaccinated as does not distinguish between infected and vaccinated horses 2. Lower reproducibility between laboratories than SRH or commercially available ELISA 3. Maybe difficult to remove nonspecific inhibitors of haemagglutination	¹ Mavadiya <i>et al.</i> (2012); ² Aharonson-Raz <i>et al.</i> (2014)

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
SRH ☑☑☐ Horse	Serum Antibodies against EIV haemagglutinin	N/A	850 horses in Brazil ¹ 464 unvaccinated horses in Spain ²	N/A	1. High reproducibility 2. Correlation between antibody titres and protection from challenge 3. Seamless quantitative assessment of the antibody level	1. May not be useful if some horses are vaccinated as does not distinguish between infected and vaccinated horses. 2. Need expertise 3. It takes longer time than HI and ELISA 4. No high-throughput	¹ Daly <i>et al.</i> (2021); ² Jurado-Tarifa <i>et al.</i> (2018)
ELISA ☑☑☐ Horse	Serum Antibodies against nucleoprotein	DSE of 99.2% (n = 475, and a DSP of 96.7% (n = 1323, (Sergeant <i>et al.</i> , 2009)	23 horses vaccinated with canarypox vaccine and experimentally challenged 217 days later 49 horses vaccinated with canarypox vaccine and not exposed to the virus ¹ Serial serum samples from 203 naturally and 14 experimentally infected horses, from 60 weanlings following primary vaccination with five different vaccines (two whole inactivated vaccines, two ISCOM-based subunit vaccines and a recombinant canarypox virus vaccine) ² 475 PCR confirmed infected horses and 1323 horses from un-infected zones in Australia ³ . 284 horses in Nigeria ⁴	N/A	1. Commercially available ELISA with no preparation of antigens required 2. Good for screening large numbers of samples – high throughput 3. Capacity to distinguish between infected horses and horses vaccinated with canarypox-vectored vaccine with HA gene only	1. Less sensitive than HI and SRH in an endemic country 2. Risk of false positive 3. Need specialised equipment	¹ Kirkland & Delbridge (2011); ² Galvin <i>et al.</i> (2013); ³ Sergeant <i>et al.</i> (2009); ⁴ Meseko <i>et al.</i> (2016)

Appendix 2: Equine influenza
Intended purpose of test: individual animal freedom from infection prior to movement

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
Real-time RT-PCR ☒ ☒ ☒ Horse	NP swab; Matrix gene	Estimated using a two-test (nucleoprotein ELISA and RT-PCR) – in-two population BLCM. ¹ DSe of RT-PCR was 99.3%. (95%PI); DSp of RT-PCR was 99.8%. (95%PI)	A total of 1248 samples from three continents. The two populations were created based on country status for equine influenza; 276 horses in populations reported to be equine influenza free and 972 horses in countries where equine influenza was circulating ¹	Validated by WOA Reference Laboratory in Ireland – report available on request	1. Very sensitive, detects both vaccinated and subclinical positive horses 2. Detects influenza type A strains including non-equine viruses that may jump the species barrier ² 3. Well established internationally 4. Rapid 5. Proficiency panel of nasal samples available from Reference Laboratory	1. Highly sensitive –positive horses are not necessarily infectious 2. “Snapshot in time” i.e. dependent on the status of the animal at a specific time point when the sample is collected 3. Possibility for contamination at sample collection or in laboratory, leading to false positives 4. Need specialised equipment	¹ Gildea <i>et al.</i> (2018) ² Heine <i>et al.</i> (2007)
Antigen-capture ELISA ☒ ☐ ☐	NP swab Nucleoprotein	Using RT-PCR as reference standard DSe of ELISA was 35% DSp was 100%	- 75 swabs from 64 horses on seven premises where EI outbreaks were confirmed - Seven samples from seven seronegative healthy foals	See reference	Rapid High specificity	1. Less sensitive than RT-PCR 2. Need specialised equipment 3. Positive horses are not necessarily infectious	Galvin <i>et al.</i> (2014)
Virus isolation ☒ ☐ ☐ Horse	NP swab Infectious virus	Using RT-PCR as reference standard DSe of VI was 9% DSp was 100% Comparing VI and an in-house nucleoprotein ELISA 75% positive by ELISA, 56% positive by VI	- 75 swabs from 64 horses on seven premises where EI outbreaks were confirmed - Seven samples from seven seronegative healthy foals ¹ - 238 swabs submitted during 1989 outbreak in UK ²	See references	1. Indicates active infection 2. Little specialist equipment required	1. Less sensitive than RT-PCR or ELISA 2. Time consuming and labour intensive 2. Slower than RT-PCR or ELISA 3. Sensitivity varies with virus strain 4. Requires embryonated eggs	¹ Galvin <i>et al.</i> (2014); ² Livesay <i>et al.</i> (1993)
RAD ☒ ☐ ☐ Horse	NP swab Influenza A viral nucleoprotein primarily for human viruses	Using RT-PCR as reference standard 22 RADs tested had DSe of 27% to 73% DSp was 100%	30 RT-PCR positive swabs from horses on eight premises where EI outbreaks were confirmed and 30 from horses in influenza free country	See a reference	1. Rapid 2. Requires little technical expertise and no specialist equipment 3. Low risk of false positives	1. Risk of false negatives 2. Large differences in sensitivity of different RADs	Yamanaka <i>et al.</i> (2016)

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
HI ☒ ☒ ☐ Horse	Paired sera Antibodies against haemagglutinin	N/A	- 23 paired sera from horses experienced EI epidemic in Argentina ¹ - 20 paired sera from horses experienced EI epidemic in Japan ²	N/A	1. Simplicity 2. Enables a diagnosis of recent infection to be reached when virus detection is negative. The comparison of paired samples collected 14–28 days apart covers a longer period than a sample collected for virus detection 3. Cost-effective 4. Measurement of antibody level	1. Time-consuming due to the need for paired sera 2. Low reproducibility between laboratories 3. Maybe difficult to remove nonspecific inhibitors of haemagglutination	¹ Perglione <i>et al.</i> (2016); ² Yamanaka <i>et al.</i> (2008)
SRH ☒ ☒ ☐ Horse	Paired sera Antibodies against EIV	N/A	- 52 paired sera from horses experienced EI epidemic in the UK ¹ - 16 paired sera from horses experienced EI epidemic in Argentina ²	N/A	1. High reproducibility 2. Correlation between antibody titres and protection from challenge 3. Seamless quantitative assessment of the antibody level 4. Enables a diagnosis of recent infection to be reached when virus detection is negative. The comparison of paired samples collected 14–28 days apart covers a longer period than a sample collected for virus detection	1. Time-consuming due to the need for paired sera 2. Need expertise 3. It takes longer to perform than HI and ELISA 4. Not high-throughput	¹ Newton <i>et al.</i> (1999); ² Perglione <i>et al.</i> (2016)
ELISA ☒ ☐ ☐ Horse	Paired sera Antibodies against nucleoprotein	In 203 naturally infected horses, sensitivity and specificity of 69% and 95%, respectively, compared with HI Sensitivity and specificity of 67% and 94%, respectively, compared with SRH	- 22 horses for vaccine and experimental challenge studies and 49 horses for vaccine study ¹ 203 naturally and 14 experimentally infected horses, 60 weanlings following primary vaccination with five different vaccines and 44 adult horses following annual booster vaccination with six different vaccines ²	N/A	1. Commercially available ELISA with no preparation of antigens required 2. Application for distinguish between infected horses and horses vaccinated with canarypox-vectored vaccine with HA	1. Less sensitive than HI and SRH in an endemic country 2. Risk of false positive 3. Need specialised equipment 4. Time-consuming due to the need for paired sera	¹ Kirkland & Delbridge (2011); ² Galvin <i>et al.</i> (2013)

Appendix 3: Equine influenza
Intended purpose of test: contribute to eradication policies

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Real-time RT-PCR</u> ☒ ☒ ☒ <u>Horse</u>	<u>NP swab;</u> <u>Matrix gene</u>	<u>Estimated using a two-test (nucleoprotein ELISA and RT-PCR) -in-two population BLCM¹ DSe of RT-PCR was 99.3%. (95%PI); DSp of RT-PCR was 99.8%. (95%PI)</u>	<u>- A total of 1248 samples from three continents. The two populations were created based on country status for equine influenza; 276 horses in populations reported to be equine influenza free and 972 horses in countries where equine influenza was circulating¹</u> <u>- More than 32,000 tests during successful eradication programme in Australia³</u>	<u>Validated by WOA Reference Laboratory in Ireland – report available on request</u>	<u>1. Very sensitive, detects both vaccinated and subclinical positive horses</u> <u>2. Detects influenza type A strains including non-equine viruses that may jump the species barrier²</u> <u>3. Well established internationally</u> <u>4. High-throughput</u> <u>5. Rapid</u> <u>6. Proficiency panel of nasal samples available from Reference Laboratory</u>	<u>1. Highly sensitive –positive horses are not necessarily infectious</u> <u>2. Possibility for contamination at sample collection or in laboratory, leading to false positives</u> <u>3. Need specialised equipment</u>	<u>¹Gildea <i>et al.</i> (2018);</u> <u>²Heine <i>et al.</i> (2007);</u> <u>³Sergeant & Wilson (2011)</u>
<u>ELISA</u> ☒ ☒ ☐ <u>Horse</u>	<u>Serum</u> <u>Antibodies against nucleoprotein</u>	<u>DSE of 99.2% (n = 475, and a DSP of 96.7% (n = 1323, (Sergeant <i>et al.</i>, 2009)</u>	<u>23 horses vaccinated with canarypox vaccine and experimentally challenged 217 days later</u> <u>49 horses vaccinated with canarypox vaccine and not exposed to the virus¹</u> <u>Serial serum samples from 203 naturally and 14 experimentally infected horses, from 60 weanlings following primary vaccination with five different vaccines (two whole inactivated vaccines, two ISCOM-based subunit vaccines and a recombinant canarypox virus vaccine)²</u> <u>475 PCR confirmed infected horses and 1323 horses from un-infected zones in Australia³</u>	<u>N/A</u>	<u>1. Commercially available ELISA with no preparation of antigens required</u> <u>2. Good for screening large numbers of samples – high throughput</u> <u>3. Capacity to distinguish between infected horses and horses vaccinated with canarypox-vectored vaccine with HA gene only</u>	<u>1. Less sensitive than HI and SRH in an endemic country</u> <u>2. Risk of false positive</u> <u>3. Need specialised equipment</u>	<u>¹Kirkland & Delbridge (2011);</u> <u>²Galvin <i>et al.</i> (2013);</u> <u>³Sergeant <i>et al.</i> (2009)</u>

Appendix 4: Equine influenza
Intended purpose of test: confirmation of clinical cases

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
Real-time RT-PCR ☒ ☒ Horse	NP swab; Matrix gene	Estimated using a two-test (nucleoprotein ELISA and RT-PCR) -in-2 population BLCM ¹ DSe of RT-PCR was 99.3%. (95%PI); DSp of RT-PCR was 99.8%. (95%PI)	A total of 1248 samples from three continents. The two populations were created based on country status for equine influenza; 276 horses in populations reported to be equine influenza free and 972 horses in countries where equine influenza was circulating ¹	Validated by WOAHI Reference Laboratory in Ireland – report available on request	1. Very sensitive, detects both vaccinated and subclinical positive horses 2. Detects influenza type A strains including non-equine viruses that may jump the species barrier ² 3. Well established internationally 4. High-throughput 5. Rapid 6. Proficiency panel of nasal samples available from Reference Laboratory	1. Highly sensitive –positive horses are not necessarily infectious 2. Possibility for contamination at sample collection or in laboratory leading to false positives 3. Need specialised equipment	¹ Gildea <i>et al.</i> (2018) ² Heine <i>et al.</i> (2007)
Antigen-capture ELISA ☒ ☐ ☐	NP swab Nucleoprotein	Using RT-PCR as reference standard DSe of ELISA was 35% DSp was 100%	- 75 swabs from 64 horses on seven premises where EI outbreaks were confirmed - Seven samples from seven seronegative healthy foals	See reference	Rapid High specificity	1. Less sensitive than RT-PCR 2. Need specialised equipment. 3. positive horses are not necessarily infectious.	Galvin <i>et al.</i> (2014)
Virus isolation ☒ ☒ Horse	NP swab Infectious virus	Using RT-PCR as reference standard DSe of VI was 9% DSp was 100% Comparing VI and ELISA 75% positive by ELISA, 56% positive by VI	- 75 swabs from 64 horses on seven premises where EI outbreaks were confirmed - Seven samples from seven seronegative foals ¹ - 238 swabs submitted during 1989 outbreak in UK ²	See references	1. Indicates active infection. 2. Little specialist equipment required	1. Less sensitive than RT-PCR or ELISA 2. Time consuming and labour intensive 2. Slower than RT-PCR or ELISA 3. Sensitivity varies with virus strain 4. Requires embryonated eggs	¹ Galvin <i>et al.</i> (2014); ² Livesay <i>et al.</i> (1993)
RAD ☒ ☒ Horse	NP swab Influenza A viral nucleoprotein primarily for human viruses	Using RT-PCR as reference standard 22 RADs tested had DSe of 27% to 73% DSp was 100%	30 RT-PCR positive swabs from horses on eight premises where EI outbreaks were confirmed and 30 from horses in influenza free country	See a reference	1. Rapid 2. Requires little technical expertise and no specialist equipment 3. Low risk of false positives	1. Risk of false negatives 2. Large differences in sensitivity of different RADs 3. Not high throughput	Yamanaka <i>et al.</i> (2016)

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>HI</u> ☒ ☒ ☐ <u>Horse</u>	<u>Paired sera</u> <u>Antibodies against haemagglutinin</u>	<u>N/A</u>	- 23 paired sera from horses experienced EI epidemic in Argentina ¹ - 20 paired sera from horses experienced EI epidemic in Japan ²	<u>N/A</u>	1. <u>Simplicity</u> 2. <u>Enables a diagnosis of recent infection to be reached when virus detection is negative. The comparison of paired samples collected 14–28 days apart covers a longer period than a sample collected for virus detection</u> 3. <u>Cost-effective</u> 4. <u>Measurement of antibody level</u>	1. <u>Time-consuming due to the need for paired sera</u> 2. <u>Low reproducibility between laboratories</u> 3. <u>Maybe difficult to remove nonspecific inhibitors of haemagglutination</u>	¹ <u>Perglione <i>et al.</i> (2016);</u> ² <u>Yamanaka <i>et al.</i> (2008)</u>
<u>SRH</u> ☒ ☒ ☐ <u>Horse</u>	<u>Paired sera</u> <u>Antibodies against EIV</u>	<u>N/A</u>	- 52 paired sera from horses experienced EI epidemic in the UK ¹ - 16 paired sera from horses experienced EI epidemic in Argentina ²	<u>N/A</u>	1. <u>High reproducibility</u> 2. <u>Correlation between antibody titres and protection from challenge</u> 3. <u>Seamless quantitative assessment of the antibody level.</u> 4. <u>Enables a diagnosis of recent infection to be reached when virus detection is negative. The comparison of paired samples collected 14–28 days apart covers a longer period than a sample collected for virus detection</u>	1. <u>Time-consuming due to the need for paired sera</u> 2. <u>Need expertise</u> 3. <u>It takes longer to perform than HI and ELISA</u> 4. <u>No high-throughput</u>	¹ <u>Newton <i>et al.</i> (1999);</u> ² <u>Perglione <i>et al.</i> (2016)</u>
<u>ELISA</u> ☒ ☐ ☐ <u>Horse</u>	<u>Paired sera</u> <u>Antibodies against nucleoprotein</u>	<u>In 203 naturally infected horses sensitivity and specificity of 69% and 95%, respectively compared with HI</u> <u>Sensitivity and specificity of 67% and 94%, respectively. Compared with SRH</u>	- 22 horses for vaccine and experimental challenge studies and 49 horses for vaccine study ¹ 203 naturally and 14 experimentally infected horses, for 60 weanlings following primary vaccination with five different vaccines and 44 adult horses following annual booster vaccination with six different vaccines ²	<u>N/A</u>	1. <u>Commercially available ELISA with no preparation of antigens required</u> 2. <u>Application for distinguish between infected horses and horses vaccinated with canarypox-vectored vaccine with HA</u>	1. <u>Less sensitive than HI and SRH in an epidemic country</u> 2. <u>Lower agreement with HI and SRH</u> 3. <u>Risk of false positive</u> 4. <u>Need specialised equipment</u> 5. <u>Time-consuming due to the need for paired sera</u>	¹ <u>Kirkland & Delbridge (2011);</u> ² <u>Galvin <i>et al.</i> (2013)</u>

Appendix 5: Equine influenza
Intended purpose of test: prevalence of infection – surveillance

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
Real-time RT-PCR ☒ ☐ ☐ Horse	NP swab; Matrix gene	Estimated using a two-test (nucleoprotein ELISA and RT-PCR) – in-two population BLCM DSe of RT-PCR was 99.3%. (95%PI); DSp of RT-PCR was 99.8%. (95%PI)	- A total of 1248 samples from three continents. The two populations were created based on country status for equine influenza; 276 horses in populations reported to be equine influenza free and 972 horses in countries where equine influenza was circulating ¹ - More than 32,000 tests applied to establishing population freedom in Australia ³	Validated by WOAHA Reference Laboratory in Ireland – report available on request	1. Very sensitive, detects both vaccinated and subclinical positive horses 2. Detects influenza type A strains including non-equine viruses that may jump the species barrier ² 3. Well established internationally 4. High-throughput 5. Rapid 6. Proficiency panel of nasal samples available from Reference Laboratory	1. Highly sensitive –positive horses are not necessarily infectious 2. Possibility for contamination at sample collection or in laboratory, leading to false positives 3. Need specialised equipment	¹ Gildea <i>et al.</i> (2018); ² Heine <i>et al.</i> , (2007) ³ Sergeant & Wilson (2011)
RAD ☒ ☐ ☐ Horse	NP swab Influenza A viral nucleoprotein primarily for human viruses	Using RT-PCR as reference standard 22 RADs tested had DSe of 27% to 73% DSp was 100%	30 RT-PCR positive swabs from horses on eight premises where EI outbreaks were confirmed and 30 from horses in influenza free country	See reference	1. Rapid 2. Requires little technical expertise and no specialist equipment 3. Low risk of false positives	1. Risk of false negatives 2. Large differences in sensitivity of different RADs 3. Not high throughput	Yamanaka <i>et al.</i> (2016)
Antigen-capture ELISA ☒ ☐ ☐	NP swab Nucleoprotein	Using RT-PCR as reference standard DSe of ELISA was 35% DSp was 100%	- 75 swabs from 64 horses on seven premises where EI outbreaks were confirmed - Seven samples from seven seronegative healthy foals	See reference	1. Rapid 2. High specificity	1. Less sensitive than RT-PCR 2. Need specialised equipment 3. positive horses are not necessarily infectious	Galvin <i>et al.</i> (2014)
HI ☒ ☒ ☐ Horse	Serum Antibodies against EIV haemagglutinin	N/A	391 horses of various ages in India ¹ 110 healthy horses on 10 farms in Israel ²	N/A	1. Simplicity 2. Widely used 3. Cost-effective 4. Quicker than SRH	1. May not be useful if some horses are vaccinated as does not distinguish between infected and vaccinated horses 2. Lower reproducibility between laboratories than SRH or commercially available ELISA 3. Maybe difficult to remove	¹ Mavadiya <i>et al.</i> (2012); ² Aharonson-Raz <i>et al.</i> (2014)

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
						<u>nonspecific inhibitors of haemagglutination</u>	
<u>SRH</u> ☒ ☒ ☐ <u>Horse</u>	<u>Serum</u> <u>Antibodies against EIV</u> <u>haemagglutinin</u>	<u>N/A</u>	<u>850 horses in Brazil¹</u> <u>464 unvaccinated horses in Spain²</u>	<u>N/A</u>	<u>1. High reproducibility</u> <u>2. Correlation between antibody titres and protection from challenge</u> <u>3. Seamless quantitative assessment of the antibody level</u>	<u>1. May not be useful if some horses are vaccinated as does not distinguish between infected and vaccinated horses.</u> <u>2. Need expertise</u> <u>3. It takes longer time than HI and ELISA</u> <u>4. No high-throughput</u>	<u>¹Daly <i>et al.</i> (2021);</u> <u>²Jurado-Tarifa <i>et al.</i> (2018)</u>
<u>ELISA</u> ☒ ☒ ☐ <u>Horse</u>	<u>Serum</u> <u>Antibodies against nucleoprotein</u>	<u>DSE of 99.2% (n = 475, and a DSP of 96.7% (n = 1323, (Sergeant <i>et al.</i>, 2009)</u>	<u>23 horses vaccinated with canarypox vaccine and experimentally challenged 217 days later</u> <u>49 horses vaccinated with canarypox vaccine and not exposed to the virus¹</u> <u>Serial serum samples from 203 naturally and 14 experimentally infected horses, from 60 weanlings following primary vaccination with five different vaccines (two whole inactivated vaccines, two ISCOM-based subunit vaccines and a recombinant canarypox virus vaccine)²</u> <u>475 PCR confirmed infected horses and 1323 horses from un-infected zones in Australia³</u> <u>284 horses in Nigeria⁴</u>	<u>N/A</u>	<u>1. Commercially available ELISA with no preparation of antigens required</u> <u>2. Good for screening large numbers of samples – high throughput</u> <u>3. Capacity to distinguish between infected horses and horses vaccinated with canarypox-vectored vaccine with HA gene only</u>	<u>1. Less sensitive than HI and SRH in an endemic country</u> <u>2. Risk of false positive</u> <u>3. Need specialised equipment</u>	<u>¹Kirkland & Delbridge (2011);</u> <u>²Galvin <i>et al.</i> (2013);</u> <u>³Sergeant <i>et al.</i> (2009)</u> <u>⁴Meseko <i>et al.</i> (2016).</u>

Appendix 6: Equine influenza

Intended purpose of test: immune status in individual animals or populations post-vaccination

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>HI</u> ☒ ☒ ☐ <u>Horse</u>	<u>Serum</u> <u>Antibodies against haemagglutinin</u>	³ 509 horses - 56% of seropositivity by ELISA, 67% by HI and 89.4% by SRH	<u>- 16 horses vaccinated with canarypox-vectored vaccine with HA¹</u> <u>- 60 weanlings with primary vaccination, 44 adult horses with booster vaccination²</u> <u>- 509 racehorses in Morocco³</u>	N/A	<u>1. Simplicity</u> <u>2. Widely used</u> <u>3. Cost-effective</u> <u>4. Measurement of antibody level</u>	<u>1. Lower reproducibility between labs</u> <u>2. Maybe difficult to remove nonspecific inhibitors of haemagglutination</u>	<u>¹El-Hage <i>et al.</i> (2013);</u> <u>²Galvin <i>et al.</i> (2013);</u> <u>³Dilai <i>et al.</i> (2018)</u>
<u>SRH</u> ☒ ☒ ☒ <u>Horse</u>	<u>Serum</u> <u>Antibodies against EIV</u>	⁴ 509 horses - 56% of seropositivity by ELISA, 67% by HI and 89.4% by SRH	<u>- 16 horses in Australia vaccinated with canarypox-vectored vaccine with HA¹</u> <u>- 438 horses on three continents vaccinated with six different vaccines²</u> <u>- 103 horses vaccinated with three vaccines³</u> <u>- 509 racehorses in Morocco⁴</u>	N/A	<u>1. High reproducibility</u> <u>2. Correlation between antibody titres and protection from challenge</u> <u>3. Seamless quantitative assessment of the antibody level</u>	<u>1. Need expertise</u> <u>2. It takes longer time than HI and ELISA</u> <u>3. No high-throughput</u>	<u>¹El-Hage <i>et al.</i> (2013);</u> <u>²Cullinane <i>et al.</i> (2020);</u> <u>³Entenfellner <i>et al.</i> (2020);</u> <u>⁴Dilai <i>et al.</i> (2018)</u>
<u>ELISA</u> ☒ ☐ ☐ <u>Horse</u>	<u>Serum</u> <u>Antibodies against nucleoprotein</u>	¹ DSe was 69% and 67%, DSp was 95% and 94% compared with HI and SRH, respectively ¹ ² 509 horses – 56% of seropositivity by ELISA, 67% by HI and 89.4% by SRH	<u>- 203 naturally infected horses, 14 experimentally infected horses, 60 weanlings with primary vaccination, 44 adult horses with booster vaccination¹</u> <u>- 509 racehorses in Morocco²</u>	See a reference	<u>1. No use of infectious agents</u> <u>2. Screening large numbers of samples</u> <u>3. Application for distinguish between infected horses and horses vaccinated with canarypox-vectored vaccine with HA</u>	<u>1. Less sensitive than HI and SRH in an epidemic country</u> <u>2. Lower agreement with HI and SRH</u> <u>3. Risk of false positive</u> <u>4. Need specialised equipment</u>	<u>¹Galvin <i>et al.</i> (2013);</u> <u>²Dilai <i>et al.</i> (2018)</u>

MEETING OF THE BIOLOGICAL STANDARDS COMMISSION

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CHAPTER 3.6.10.

GLANDERS AND MELIOIDOSIS

SUMMARY

Description and importance of the disease: *Glanders is a highly contagious and fatal disease of horses, donkeys, and mules caused by infection with the bacterium Burkholderia mallei. ~~The~~ It is an obligate mammalian pathogen that spreads by direct host-to-host transmission and causes nodules and ulcerations in the skin, lymph nodes, upper respiratory tract and lungs. The skin-cutaneous form is known as 'farcy'. Melioidosis is an infectious disease, caused by B. pseudomallei. Melioidosis is uncommon, is an infectious disease with a wide host range. Unlike B. mallei, B. pseudomallei is an environmental pathogen present in humans and animals, soil and sometimes resembles glanders water. Human and animal cases of melioidosis have been reported mainly in South-East Asia and Australia, but the disease is now emerging in Africa and the Americas. This chapter focuses on the disease in horses. B. mallei and B. pseudomallei infections in equidae, which share clinical similarities due to their genetic proximity. Burkholderia mallei has evolved from B. pseudomallei by genomic reduction of genetic information and is phylogenetically considered as a phylogenetic clone, i.e. a pathovar of B. pseudomallei.*

Control of glanders and melioidosis requires testing of suspect clinical cases, screening of ~~apparently normal-subclinical~~ equids, and ~~elimination-culling of reactors. Stable-confirmed cases.~~ Good stable hygiene and manure management are ~~imperative-essential for disease control~~. As B. mallei and B. pseudomallei can be transmitted to humans, all ~~infected or contaminated (or potentially infected or contaminated)~~ material biological samples must be handled in a laboratory with appropriate biosafety and biosecurity controls following a biorisk analysis.

Detection of the agent: Smears from fresh ~~material-specimens~~ containing B. mallei ~~bacteria or B. pseudomallei~~ may reveal Gram-negative nonsporulating, nonencapsulated rods. Electron microscopy reveals a capsule-like structure in both bacteria. Burkholderia mallei grows aerobically and prefers ~~media that contain glycerol. Standard-containing media, for isolation of Burkholderia pseudomallei can be used grow on standard media~~ and selective enrichment techniques have been developed. ~~The presence of a capsule-like cover has been demonstrated by electron microscopy in both agents. Unlike the Pseudomonas species and the closely related bacterium B. pseudomallei, B. mallei is nonmotile. For identification, Biochemical phenotyping can be used for identification. Commercially available biochemical identification kits lack diagnostic sensitivity. MALDI-TOF¹ spectra have been made are available for both agents in the past years. Whole genome sequences have been published. Specific monoclonal antibodies and polymerase chain reaction (PCR), as well as real-time PCR assays are available. Whole genome sequences have been published for both bacteria.~~

Serological tests: Complement fixation is an accurate and reliable serological method for ~~diagnostic use in the diagnosis of glanders in equids and is the test recommended for individual animal freedom from infection prior to movement alongside the newly validated recombinant double-antigen enzyme-linked immunosorbent assays (ELISA).~~ Other ELISAs are promising but require further validation ~~glanders in equids. A rose bengal plate agglutination test for glanders has been developed. An immunoblot test based on a crude formalin preparation of B. mallei antigens from isolates of from~~

1 MALDI-TOF: Matrix assisted laser desorption ionisation time of flight [mass spectrometry]

different geographical regions is also a sensitive and specific assay. None of these tests may also be positive in horses with melioidosis. Enzyme-linked immunosorbent assays show promise for specific diagnosis in equids once their validation is complete can distinguish between infections caused by *B. mallei* and *B. pseudomallei*. Furthermore, the occurrence of *B. pseudomallei* in tropical soils may influence the results and must be considered.

Mallein test: The mallein test is a hypersensitivity skin test against *B. mallei*. The test is not generally recommended because of animal welfare concerns, however it can but may be useful in remote endemic areas where sample transport or proper cooling of samples is not possible. Mallein involves an intradermo-palpebral injection of mallein, a water-soluble protein fraction of the organism, is injected intradermo-palpebrally. In *B. mallei*, which causes eyelid swelling in infected animals, the eyelid swells markedly within 1–2 days. This test may also be positive in Horses with melioidosis may also test positive.

Requirements for vaccines and diagnostic biologicals: There are No vaccines are available. A mallein purified protein derivative is commercially available.

A. INTRODUCTION

Glanders is a bacterial disease of that primarily affects perissodactyls or odd-toed ungulates, such as horses, donkeys and mules. It is a zoonotic disease and has been known-recognised since ancient times. It is caused by the bacterium *Burkholderia mallei* (Yabuuchi et al., 1992) and has previously been variously classified in the past genera such as *Pseudomonas*, *Pfeifferella*, *Loefflerella*, *Malleomyces* or *Actinobacillus*. It is a serious contagious disease in Although equids and outbreaks may also occur in are the primary hosts, glanders can infect other species. Outbreaks have been reported in wild felids living in the wild or in zoological gardens and zoo animals. Susceptibility to glanders has been proven-demonstrated in camels, small ruminants, bears, wolves and dogs. Carnivores may become infected by eating, often through ingestion of infected meat, but cattle, and pigs are resistant. Small ruminants may be infected if kept in or close contact with glanderous horses (Wittig et al., 2006). Glanders generally takes an acute form in donkeys and mules with high fever and respiratory signs (swollen nostrils, dyspnoea, and pneumonia) and death occurs within a few days. In horses, glanders generally takes a more chronic course and horses may survive for several years. Chronic and subclinical 'occult' cases infected animals. Cattle, pigs, rats and birds are potential sources of infection due to the permanent or intermittent shedding of bacteria relatively resistant (Wittig et al., 2006). Khan et al. (2013) reviewed the disease, its epidemiology, diagnosis and control.

In horses-equids, inflammatory pustules and ulcers develop in the nasal conchae and nasal septae, which give rise to-septa, producing a sticky yellow discharge, accompanied by enlarged firm submaxillary lymph nodes. Stellate scarring follows upon healing of the ulcers. In the lungs, the formation of reddish nodular abscesses with a central grey necrotic zone in the lungs is accompanied by progressive debility, febrile episodes, coughing weakness, fever, cough and dyspnoea. Diarrhoea and polyuria can also occur. In the skin-cutaneous form ('farcy'), the lymphatic vessels are enlarged and 0.5–2.5 cm sized nodular abscesses ('buds') develop, which ulcerate and discharge yellow oily pus. Dry ulcers may also develop. Pyogranulomatous nodules are sometimes found in the liver and spleen. Discharges from the respiratory tract Respiratory and skin-cutaneous discharges are infective, infectious and transmission between animals, which is facilitated occurs by close contact, inhalation, ingestion of contaminated material (e.g. from infected feed and water troughs), or by inoculation (e.g. via a harness) is common through equipment such as harnesses. The incubation period can vary from days to months (Wittig et al., 2006). In horses, the disease is often chronic and can persist for years. Chronic and subclinical 'occult' cases pose a risk due to intermittent or permanent bacterial shedding (Wittig et al., 2006). In donkeys and mules, glanders typically presents acutely with high fever and respiratory signs (swollen nostrils, dyspnoea, and pneumonia), leading to death within days. Khan et al. (2013) reviewed the disease, its epidemiology, diagnosis and control. The incubation period can range from a few days to many months (Wittig et al., 2006).

Glanders is transmissible-transmitted to humans by-through direct contact with diseased-sick animals or with infected or contaminated material. In the untreated-Without treatment, acute disease, cases have a the-mortality rate can reach 95% within 3 weeks-of up to 90% (Neubauer et al., 1997 Van Zandt et al., 2013). However, survival is possible if the infected person is treated early and aggressively treatment with multiple systemic antibiotic therapies antibiotics can improve survival. A chronic form with abscessation-can abscess formation may occur (Neubauer et al., 1997).

Glanders has been eradicated from many countries by-statutory-through compulsory testing, culling of infected animals, and import restrictions. However, it persists in numerous Asian, African-parts of Asia, Africa and South American-America. It is re-emerging in countries in the Middle East, India, Pakistan, Nepal, and can be considered a re-emerging disease-Brazil. Glanders can be introduced into glanders-disease-free areas by movement of infected equids (Neubauer et al., 2005).

Melioidosis is caused by *B. pseudomallei* is the causative agent of melioidosis, a soil-dwelling bacterium historically endemic to South-East Asia (Cambodia, Indonesia, Laos, Malaysia, Myanmar, Singapore, Thailand, and originates from-Vietnam) and northern Australia. Its range is expanding and now includes the Pacific, South-East Asia (areas

within latitudes 20°N, Africa, and 20°S) the Americas (Limmathurotsakul *et al.*, 2016). It is an important soil bacterium, denitrifying organic materials and is ubiquitous in those areas. Thus, *B. Burkholderia pseudomallei* is stable in the environment. It is and it also naturally resistant to various antibiotics and disinfectants (Currie, 2015; O'Connell *et al.*, 2009; Sprague & Neubauer, 2004). *Burkholderia pseudomallei* has is an extremely broad opportunistic pathogen with a wide host range, including wildlife, farm animals and humans (Rush & Thomas, 2012). *Burkholderia pseudomallei* has regularly been isolated, mammals, birds and reptiles (Birnie *et al.*, 2022).

In humans, melioidosis can cause a wide range of signs, from abscesses in various organs (spleen, liver, lung, lymph nodes), from milk in cases with mastitis, or from faeces in diarrhoea cases. This chapter focuses on localised infections to severe systemic disease. Despite timely and aggressive multi-antibiotic therapy, the disease in horses, which may closely resemble glanders, but more diverse presentations also occur is often fatal (Meumann *et al.*, 2024).

Melioidosis in horses has is rare and often presents with signs similar to glanders. Occurrences have been reported as peracute cases with high fever, septicaemia, limb oedema, diarrhoea and death occurring within 24 hours, or acute cases with limb oedema, slight mild colic and intestinal hypermotility. More typically, melioidosis runs has a subacute to chronic course from of 3 weeks to 3 months with no without loss of appetite. Further signs Other reported are signs include emaciation, oedema and lymphangitis of the limbs, mild colic, diarrhoea, pneumonia, cough and nasal discharge. Skin involvement may initially resemble fungal eczema, later becoming papular without abscess formation. Acute meningoencephalitis and keratitis may be seen occur. If the infection is acquired *per os*, intestinal signs may predominate. It is reported that The lungs are always affected and show reported to be involved with signs of acute bronchopneumonia and numerous multiple abscesses. Severe enteritis, multiple microabscessation in the kidneys, and necrotic foci in liver and spleen may be found at necropsy. Ulcers on the mucosa of the upper respiratory tract and cicatricial scar tissue on the septum nasi and epiglottis may be found. Ulcers and nodules in the skin and subcutis of the limbs may be confused with farcy. Conjunctivitis and keratitis, although rare, have been reported, though rare. The incubation period for naturally occurring infection in animals is not known. Spread unknown. Experimental infection of horses with *B. pseudomallei* (via oral or subcutaneous routes) failed to generate lesions in the nasal septum or conchae; however, typical pyogranulomas indistinguishable from those caused by glanders were observed in the lungs and other organs, and the pathogen was detected in the bladder (Wernery *et al.*, 2019a; 2019b). The disease may be spread by infected animals or by contaminated materials in the animals' their environment. Infection is thought to occur by inoculation, ingestion or inhalation of environmental organisms. Inapparent infection may occur (reviewed by Sprague & Neubauer, 2004). In humans the disease is frequently fatal despite timely institution of therapy (Currie, 2015; Kingsley *et al.*, 2016b). No approved antibiotic regimens for horses have been are recommended for horses.

Melioidosis can be considered an emerging disease and is present in many regions worldwide (Limmathurotsakul *et al.*, 2016). Control In regions where B. pseudomallei is not endemic (e.g. non-tropical countries), control of melioidosis requires testing of suspected clinical cases, screening of apparently normal equids, and elimination of reactors. In areas with a temperate climate, special thought has to be given to Emphasis should be placed on maintaining stable hygiene practices (disinfection, removal of faeces several times a day, reduction of the reduced use of water for cleaning, disinfection of hooves and lower limbs, movement control) and soil hygiene. Suitable methods to prevent spread by treatment of manure, waste water or rodent control have not been investigated or reported (Dodin, 1992). Dodin (1992) stated that The outbreak at the enzootics Jardin des Plantes in France declined and in the infected soils were cleared only after all 1970s highlighted the risks of importing infected animals were removed. Consequently, Sprague & Neubauer (2004) proposed that equids with melioidosis should be culled immediately in non-endemic countries to prevent local establishment, which led to the contamination of environmental reservoirs that would pose an ongoing risk of infection for zoo animals, humans and animals nearby cavalry horses, facilitating the spread of the disease across the country. It took a decade to bring the epidemic under control. In addition, the infection can be imported from species other than equidae. When handling suspect or known infected animals or fomites, stringent precautions must be taken suspected or confirmed to prevent self-infection or transmission of the bacteria. Laboratory samples must be infected with B. mallei or B. pseudomallei, or when handling potentially contaminated materials, strict biosafety protocols are essential. This includes securely packaged, kept packaging specimens to prevent spillage, keeping specimens cool (not frozen) and shipped as outlined during transport, and following the transport guidelines for biological materials in Chapter 1.1.3 *Transport of biological materials*. All manipulations with of potentially infected/contaminated material must be performed at an appropriate biosafety and containment level as determined by a biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

Outside its host, *B. mallei* has limited resistance to environmental factors such as desiccation, heat, light or and chemicals, making its survival beyond 2 weeks is unlikely (Neubauer *et al.*, 1997). However, it can persist for several months under favourable conditions, and can remain viable in tap water for at least 1 month. Effective disinfectants include benzalkonium chloride (1/2,000), sodium hypochlorite (500 ppm available chlorine), iodine, mercuric chloride in alcohol, and potassium permanganate. Phenolic disinfectants are less effective (St. Georgiev, 2008). In contrast, *B. pseudomallei* is more resilient, surviving up to 16 years in water (Pumpuang *et al.*, 2011), 7 months in muddy water and up to 30 months in laboratory soil. Chlorine has only a bacteriostatic effect on *B. pseudomallei*, with bacteria recoverable even in water containing up to 1000 p.p.m. free chlorine (review: Sprague & Neubauer, 2004).

B. DIAGNOSTIC TECHNIQUES

Test methods available for the diagnosis of glanders and their purpose are listed in Table 1. For melioidosis, *B. pseudomallei* can be specifically confirmed by culture and polymerase chain reaction (PCR). Although serological tests developed for glanders may cross-react and give an indication of potential melioidosis cases, these tests are not fully validated for melioidosis. This issue is particularly relevant in areas where *B. pseudomallei* is present in the soil, as environmental exposure may lead to positive test results.

Table 1. Test methods available for the diagnosis of glanders 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
Detection of the agent						
Conventional PCR	□□□	□□□	□□□	☑□□-☑☑□	□□□	□□□
Real-time PCR	□□□	□□□	□□□	☑☑☑	□□□	□□□
Culture	□□□	□□□	□□□	☑□□-☑☑□	□□□	□□□
Detection of immune response						
Complement fixation ^(f)	☑☑□	☑☑□ ^(g)	☑☑☑	☑☑□	☑☑☑	□□□
ELISA ^(g,h)	☑□□-☑☑□	☑□□-☑☑□	☑☑□-☑☑☑	☑□□-☑☑□	☑☑□-☑☑☑	□□□
Recombinant double-antigen ELISA	☑☑☑	☑☑☑	☑☑☑	☑☑☑	☑☑☑ ^(h)	□□□
Mallein skin test	☑□□	☑□□	☑□□	☑□□	☑□□	□□□
Western blotting	☑□□	☑□□	☑☑□	☑□□	☑☑□	□□□

Key: ☑☑☑ = recommended for this purpose; ☑☑□ = recommended but has limitations;

☑□□ = suitable in very limited circumstances; □□□ = not appropriate for this purpose.

PCR = polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay.

^(a)Horse samples only – care needed with interpretation of test on donkey samples. See Appendix 1 of this chapter for justification table for the scores given to the tests for this purpose.

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

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

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

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

^(f)Horse samples only – care needed with interpretation of test on donkey samples.

^(g)Only validated ELISAs should be used, especially for mules and donkeys, which are not always included in validation studies.

^(h)Special consideration given in tropical regions where *B. pseudomallei* is present in soils.

For melioidosis, culture and polymerase chain reaction (PCR) methods may be used to detect and identify the organism for confirmation of clinical cases, as described in the text below, but serological methods are not yet available for this infection.

1. Interpretation of tests for the diagnosis of glanders

Confirmation of a diagnosis of glanders should be based on the or melioidosis requires isolation and identification of *Burkholderia-B. mallei* in a sample or *B. pseudomallei* from an equid sample or a derived-product derived from that equid, or the identification in such samples, or detection of antigen or their specific genetic material specific to *B. mallei*. Supporting evidence may be provided by in these samples. In animals showing clinical signs, a positive serological test results such as a titre of 1/5 in the complement fixation test (CFT) result, confirmed by a second

test with of equal or higher greater sensitivity and higher specificity, e.g. *B. mallei* specific lipopolysaccharide (LPS)-western blot, I enzyme-linked immunosorbent assay (ELISA) (indirect) (based on a recombinant protein from type VI secretion system) or C-ELISA (competitive-ELISA) (based on *B. mallei* specific monoclonal antibodies may indicate glanders or melioidosis and should prompt further investigation for direct detection of the agent. The serological tests to be used depend on the prevalence of the disease (Sprague et al., 2009).

2. Detection of the agent

Cases ~~for requiring specific glands~~ investigation for glanders or melioidosis should be clinically differentiated on clinical grounds from other chronic infections affecting of the nasal mucous membranes, sinuses or the skin. ~~Among these are~~ These include strangles (*Streptococcus equi*), ulcerative lymphangitis (*Corynebacterium pseudotuberculosis*), pseudotuberculosis (*Yersinia pseudotuberculosis*) and sporotrichosis (*Sporotrichium* spp.). Glanders should be excluded from suspected cases of epizootic lymphangitis (*Histoplasma capsulatum farciminosum*), with which it has many clinical similarities. ~~In horses and humans in particular, glanders should be distinguished from melioidosis.~~

2.1. Morphology of *Burkholderia mallei* and *B. pseudomallei*

Burkholderia mallei ~~organisms are fairly numerous is often found~~ in smears from fresh lesions, but ~~scarce less commonly~~ in older lesions. Smears ~~should can~~ be stained with methylene blue or Gram's stain. The Gram-negative rods have rounded ends, are 2–5 µm long and 0.3–0.8 µm wide with granular inclusions of various size. The bacteria are generally located extracellularly and frequently stain irregularly and poorly when Gram's stain is used. They do not have a readily visible capsule under the light microscope and do not form spores. The presence of a capsule-like ~~cover coat~~ has been ~~verified observed~~ by electron microscopy. This capsule is composed of neutral carbohydrates and serves to protect the cell from ~~unfavourable adverse~~ environmental factors. Unlike other organisms in the *Pseudomonas* group and its close relative *B. pseudomallei*, *B. mallei* has no flagellae and is therefore nonmotile (Sprague & Neubauer, 2004). Nonmotility is the most important phenotypic characteristic ~~diagnostically for diagnosis~~ and must be demonstrated when pure culture is available. The organisms are difficult to detect in tissue sections, where they may have a beaded appearance. In culture media, ~~they vary in their appearance depending on varies according to~~ the age of the culture and ~~the~~ type of medium. ~~In Older cultures, there is much pleomorphism are highly pleomorphic.~~ Branching filaments form on the surface of broth cultures (Neubauer et al., 2005).

In contrast, *B. pseudomallei* is motile due to the presence of flagella and can be found in both acute and chronic lesions. These Gram-negative rods are 2–4 µm long and 0.4–0.8 µm wide, with rounded ends. Smears from lesions or cultures can be stained with Gram's stain or methylene blue. *Burkholderia pseudomallei* is primarily extracellular, but may be present within macrophages during infection. It has a polysaccharide capsule that aids in immune evasion and survival in the environment, although this capsule is not readily visible under the light microscope. In tissue sections, *B. pseudomallei* may appear granular or clumped. On solid media, the colony morphology of *B. pseudomallei* varies, with some strains forming smooth colonies and others developing a wrinkled appearance, depending on the strain and culture conditions.

2.2. Cultural characteristics

It is preferable to attempt isolation from unopened ~~Isolation of *B. mallei* is most effective when samples are taken from fresh, uncontaminated lesions. to avoid bacterial overgrowth. Key organs include the lungs, liver, spleen and lymph nodes in the vicinity of the lesions. The nasal cavity, trachea and skin lesion (if present) should also be sampled.~~

Burkholderia mallei is an aerobic and facultative anaerobic only bacterium that can grow anaerobically in the presence of nitrate, ~~growing optimally at 37°C. It grows well, but best, albeit slowly, at 37°C. It can be grown on a variety of on-culture media, including sheep blood agar. Incubation of cultures for 72 hours is~~ The recommended; glycerol incubation period is 72 hours. Glycerol enrichment is particularly useful. The tiny greyish shiny-shining colonies of *B. mallei* on sheep blood agar can be easily overgrown by other bacteria; hence so careful observation is needed to not to overlook miss the bacteria after 72 hours of incubation. After a few days on glycerol agar, a confluent, smooth, moist and slightly viscous cream-coloured growth can be is observed. On continued further incubation, the growth thickens and becomes dark brown and tough. *Burkholderia mallei* also grows well on glycerol potato agar and in glycerol broth, on which forming a slimy pellicle forms. Growth on nutrient agar, the growth is much less effusive profuse, and growth is poor on gelatine. Various is poor. Several commercially available *Burkholderia* selective agars enable allow the growth of *B. mallei* (Glass et al., 2009).

Even in fresh samples obtained under sterile conditions, *B. mallei* is often overgrown by other bacteria, which makes making isolation extremely difficult (Wernery, 2009).

Confirmation of the identity of suspected isolates is by biochemical reactions or by PCR. Growth characteristics may alter *in vitro*, so fresh isolates should be used for identification reactions. The positive biochemical reactions include reduction of nitrates, utilisation of arginine by arginine dihydrolase, assimilation of glucose, N acetyl glucosamine and gluconate. Strain to strain variation is observed in the assimilation reactions of arabinose, fructose, mannose, mannitol, adipic acid, malate, trisodium citrate, phenyl acetic acid and VP reaction, which needs an incubation time of 48 hours. Indole is not produced, horse blood is not haemolysed and no diffusible pigments are produced in cultures. Various biochemical characteristics can be used for the differentiation of *B. mallei* from *B. pseudomallei*, as *B. mallei* has lost many fermentative activities during phylogeny (Neubauer *et al.*, 1997). Commercially available laboratory biochemical identification systems can be used for confirmation that an organism belongs to the *Pseudomonas* group. In general, however, commercially available systems are not suitable for unambiguous identification of members of the steadily growing number of species within the genus *Burkholderia* (Glass & Popovic, 2005; Homarajata *et al.*, 2016; Inglis *et al.*, 2005; Kingsley *et al.*, 2016a; Lau *et al.*, 2015; Zong *et al.*, 2012).

A novel selective medium has been described for the isolation of *B. mallei* from equine specimens (Kinoshita *et al.*, 2019). This agar was prepared by combining 5 g of proteose peptone no. 3, 10 g of tryptone pancreatic digest of casein, 0.25 g of magnesium sulfate, 1 g of sodium pyruvate, 5 g of sodium chloride, 40 ml of glycerol, 3 mg of crystal violet (3 ml from 0.1% w/v crystal violet in distilled water), 50 mg of cycloheximide (5 ml from 1% [w/v] cycloheximide in absolute ethanol), 15 g of agar, and distilled water (960 ml). The mixture is sterilised at 121°C for 15 minutes. After cooling to 45°C, the medium is supplemented with three antimicrobials: 16.7 mg of ticarcillin disodium salt (668 µl from 25 mg/ml stock solution), 197.8 mg of fosfomycin sodium (1520 µl from 130 mg/ml stock solution), 40,000 units of polymyxin B (400 µl from 100,000 units/ml stock solution). For heavily Lack of motility is therefore of special relevance. Cell matrix assisted laser desorption/ionisation mass spectrometric typing can be used (Cunningham & Patel, 2013; Karger *et al.* 2012). A bacteriophage specific for *B. mallei* is available. Reference laboratories or specialised laboratories use *B. pseudomallei* specific antibodies that are not commercially available and that have not been validated for use in equids (Sprague & Neubauer, 2004).

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

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

Although there are no validated procedures methods for the isolation of isolating *B. pseudomallei* from horse equine samples exist. Cultivation may be successful from samples of, cultivation from ulcers, lesions or excretions. Standard media, i.e. urine can be successful (Wernery *et al.*, 2019a). Initial isolation can be performed on blood agar or MacConkey agar, are used for isolation of *B. pseudomallei* and selective enrichment techniques e.g. Selective media such as Ashdown agar, Galimand's broth or *B. pseudomallei* selective agar (and BPSA) have been developed (Limmathurotsakul *et al.*, 2012; improve detection (see website [Meloidosis - Home](#)) (Peacock *et al.*, 2005; Prakash *et al.*, 2014; Roesnita *et al.*, 2012; Sprague & Neubauer, 2004; Trung *et al.*, 2011). Colony morphology varies from). *Burkholderia pseudomallei* colonies may appear smooth to rough depending on the medium and strain, and often becomes or wrinkled after, often with a few days of incubation. A metallic sheen over the area of confluent growth on Ashdown or Columbia agar is an important feature.

All culture media must be quality controlled to ensure growth of the suspect organism from samples with low bacterial loads.

Biochemical tests such as the API Gallery are useful for the presumptive identification of *B. pseudomallei* (Dance *et al.*, 1989) *B. mallei* and *B. pseudomallei*. The API 20NE system is used to identify non-enteric, oxidase-positive bacteria. *Burkholderia mallei* and *B. pseudomallei* share several similar and different

biochemical characteristics. Typical positive results for *B. mallei* include nitrate reduction, oxidase activity and arginine dihydrolase activity. It also shows positive assimilation of glucose, N-acetylglucosamine and gluconate. However, *B. mallei* does not produce urease, which may help to distinguish it from *B. pseudomallei* (Neubauer *et al.*, 1997). In contrast, *B. pseudomallei* also shows positive nitrate reduction, oxidase activity and glucose assimilation. It typically has positive or variable arginine dihydrolase activity. Unlike *B. mallei*, *B. pseudomallei* is urease positive, a key differentiator between the two bacteria. Although commercial biochemical systems can confirm membership of the *Pseudomonas* group, they are generally not reliable for accurate identification of the diverse *Burkholderia* genus (Glass & Popovic, 2005; Inglis *et al.*, 2005; Lau *et al.*, 2015; Zong *et al.*, 2012). These results should be interpreted alongside other identification methods, such as MALDI-TOF MS² (Cunningham & Patel, 2013; Karger *et al.* 2012), which is a more advanced technique, although database inconsistencies can occasionally lead to misidentifications, or PCR, which remains the most reliable method for accurately distinguishing between the two species.

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

Burkholderia pseudomallei can survive in water up to 16 years (Pumpuang *et al.*, 2011), in muddy water for up to 7 months and in soil in the laboratory for up to 30 months. Chlorine has only a bacteriostatic effect on the agent as bacteria were recovered from water containing up to 1000 p.p.m.

National regulations and guidelines for should be observed for the safe handling and application of disinfectants should be observed.

2.3. Identification of *Burkholderia mallei* and *B. pseudomallei* by polymerase chain reaction (PCR) and real-time PCR

PCR methods available for *B. mallei* and *B. pseudomallei* are listed in Tables 2 and 3.

Table 2. Conventional PCR methods available for *Burkholderia mallei* and *B. pseudomallei*

Pathogen/ target gene	Primer (5'–3')	Concentration of working solution	Cycling parameters ^(a)
Method 1: Scholz <i>et al.</i> (2006); GenBank Accession No.: NC_006350, NC_006351; amplicon size: 989bp			
<i>Burkholderia mallei</i> /fliP	Bma-IS407-fliP-f: 5'-TCA-GGT-TTG-TAT-GTC-GCT-CGG-3' Bma-fliP-r: 5'-CTA-GGT-GAA-GCT-CTG-CGC-GAG-3'	10 µM 10 µM	94°C/10 min, 35 × (94°C/30 sec, 65°C/30 sec, 72°C/60 sec); 72°C/7 min

^(a)Conditions of used polymerases and mastermixes must be considered.

Table 3. Real-time PCR methods available for *Burkholderia mallei* and *B. pseudomallei*

Pathogen/ target gene	Primer/probe (5'–3')	Concentration of working solution	Cycling parameters ^(a)
Method 1: Tomaso <i>et al.</i> (2006); GenBank Accession No.: NC_006350, NC_006351			
<i>Burkholderia mallei</i> /fliP	Bma-flip-f: CCC-ATT-GGC-CCT-ATC-GAA-G Bma-flip-r: GCC-CGA-CGA-GCA-CCT-GAT-T probe Bma-flip: FAM-CAG-GTC-AAC-GAG-CTT-CAC-GCG-GAT-C-BHQ	10 µM 10 µM 10 µM	(50°C/2 min) ^(b) , 95°C/10 min; 45 × (95°C/25 sec, 63°C/60 sec)
Method 1: Thibault <i>et al.</i> (2004); GenBank Accession No.: AF074878			
<i>Burkholderia pseudomallei</i> /orf11	orf11F: ATC-GCC-AAA-TGC-CGG-GTT-TC orf11R: CAA-ATG-GCC-ATC-GTG-ATG-TTC orf11pro: FAM-TCG-GCG-AAC-GCG-ATT-TGA-TCG-TTC-C-BHQ	50 µM 50 µM 20 µM	(50°C/2 min) ^(b) , 95°C/10 min; 45 × (95°C/10 sec, 60°C/45 sec)

^(a)Conditions of used polymerases and mastermixes must be considered. ^(b)Uracyl N-glycosylase pre-treatment in association with inclusion of uracil instead of thymine in the master mix helps to prevent contamination from previous amplifications.

Burkholderia pseudomallei and *B. mallei* are closely related bacteria with different environmental and disease profiles. *Burkholderia pseudomallei* has a highly flexible genome, influenced by frequent gene transfers and rearrangements. In contrast, *B. mallei* has evolved into a strict mammalian pathogen through genome reduction, resulting in a more uniform population, although it is still evolving.

Several PCR and real-time PCR assays have been developed for the specific detection and identification of *B. mallei* and differentiation of *B. pseudomallei* have been developed (reviewed by Lowe *et al.*, 2014). One A conventional PCR and one a real-time PCR assay were have been evaluated using samples from a natural outbreak of glanders in horses (Scholz *et al.*, 2006; Tomaso *et al.*, 2006). These two assays are therefore described presented in more detail, but in this chapter, although real-time PCR is recommended because of its higher analytical sensitivity. Inter-laboratory studies are still needed to confirm the their robustness of these assays. The guidelines and precautions outlined in Chapter 1.1.6 Principles and methods of validation of diagnostic assays for infectious diseases of terrestrial animals should be observed followed.

2.3.1. DNA preparation

Single-From agar cultures, single colonies are transferred from an agar plate to 200 µl of deionised water. After heat inactivation (for example 99–100°C for 30–20 minutes), the DNA isolation supernatant obtained after centrifugation at 8000 g for 10 minutes can be performed using commercial DNA preparation kits for gram-negative bacteria (see Scholz *et al.*, 2006 and Tomaso *et al.*, 2006). Alternatively, heat inactivated bacteria from pure cultures (eg 99°C, 10 minutes) can be used directly for PCR or subjected to DNA extraction using commercial kits designed for Gram-negative bacteria.

Tissue samples from horses (e.g. skin, lung, mucous membrane of the nasal conchae and septae, liver and spleen) that have been inactivated and preserved in formalin can be processed for DNA extraction using commercially available kits. Formalin-fixed tissues (48 hours, 10% v/v) are should be cut with a scalpel into pieces of 0.5 × 0.5 cm (approximately 500 mg). The specimens are washed twice in deionised water (10 ml), and incubated overnight in sterile saline at 4°C and minced by freezing. Samples are then frozen in liquid nitrogen, followed by grinding-crushed with a mortar and pestle, and total DNA is prepared from 50 mg tissue using a commercial extraction extracted using kits according to the manufacturer's instructions. DNA is eluted with 80 µl dH₂O or as appropriate for the kit used.

For *B. pseudomallei*, Kits have been evaluated for the DNA extraction of DNA from tissue samples formalin-fixed, paraffin-embedded tissues for the detection of *B. pseudomallei* (Obersteller *et al.*, 2016).

2.3.2. PCR assay (Scholz *et al.*, 2006) assays for *B. mallei* detection

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

The oligonucleotides used by Scholz *et al.*, (2006) are based on the differences between the *fljP* sequences from *B. mallei* ATCC 23344[†] (accession numbers NC_006350, NC_006351) and *B. pseudomallei* K96243 (accession numbers NC_006348, NC_006349). Primers Bma IS407-flip-f (5'-TCA-GGT-TTG-TAT-GTC-GCT-CGG-3') and Bma IS407-flip-r (5'-CTA-GGT-GAA-GCT-CTG-CGC-GAG-3') are used to amplify a 989 bp fragment. The PCR uses 50 µl ready to go master mix, 15 pmol of each primer, and 4 µl of template DNA. Thermal cycling conditions are 94°C for 30 seconds and 35 cycles at 65°C for 30 seconds and 72°C for 60 seconds and succeeded by a final elongation step at 72°C for 7 minutes. Visualisation of the products takes place under UV light after agarose gel (1% w/v in TAE buffer) electrophoresis and staining with nucleic acid stain. No template controls containing PCR grade water instead of template and positive controls containing *B. mallei* DNA have to be included in each run to detect contamination by amplicons of former runs or amplification failure. The lower detection limit of this assay is 10 fg or 2 genome equivalents.

Scholz *et al.* (2006) developed a conventional PCR method targeting differences in the *fljP* gene to specifically differentiate *B. mallei* from *B. pseudomallei* (Table 2). The PCR reaction contains *Taq* polymerase, primers and DNA template. Amplicons are stained with a nucleic acid dye before visualisation under UV light on agarose gels. No-template and *B. mallei* positive controls ensure reliability.

2.3.3. Real-time PCR assay (Tomaso *et al.*, 2006)

~~The assay should be. This method was then adapted to the for real-time PCR instrument used, e.g. the cycling vials should be chosen according to the manufacturer's recommendations, the concentration of the oligonucleotides may have to be increased, or the labelling of the probes altered.~~

~~The oligonucleotides used by Tomaso *et al.* (2006) are based on differences in the *fliP* sequences of *B. mallei* ATCC 23344^T (accession numbers NC_006350, NC_006351) and *B. pseudomallei* K96243 (accession numbers NC_006348, NC_006349). The fluorogenic by Tomaso *et al.* (2006, Table 2) and incorporates a probe is synthesised with 6-carboxy-fluorescein (FAM) at the 5' end and black hole quencher 1 (BHQ1) at the 3' end. Oligonucleotides used were Bma flip f (5'-CCG-ATT-GGC-CCT-ATC-GAA-G-3'), Bma flip r (5'-GCC-CGA-CGA-GCA-CCT-GAT-T-3') and probe Bma flip (5'-6FAM-CAG-GTC-AAG-GAG-CTT-CAG-GCG-GAT-C-BHQ1-3') that binds to the amplified fragment, increasing the sensitivity and specificity of the test compared with conventional PCR. The 25 µl reaction mixture volume consists of 12.5 µl 2× master mix, 0.1 µl of each primer (10 pmol/µl), 0.1 µl of the probe (10 pmol/µl) and 4 µl template DNA. Thermal-cycling conditions are 50°C for 2 minutes; 95°C for 10 minutes; 45 cycles at 95°C for 25 seconds and 63°C for 1 minute. Possible contaminations with amplification products from former reactions are inactivated by an initial A pre-incubation step using with uracil N-glycosylase. The authors suggest including an N-glycosylase (50°C for 2 minutes) and the use of uracil instead of thymine in the master mix helps to prevent contamination from previous amplifications. An internal PCR inhibition control based on a bacteriophage lambda gene target (Lambda F [5'-ATG-CCA-CGT-AAG-CGA-AAC-A-3'] Lambda R [5'-GCA-TAA-ACG-AAG-CAG-TCG-AGT-3'], Lam YAK [5'-YAK-ACG-TTA-CGG-AAA-TCG-GTA-CGG-ATA-CGG-C-DB-3']), which can be titrated to provide reproducible cycle threshold values. However, depending on the sample material a PCR targeting a housekeeping gene may be used additionally or as an alternative. No template controls containing 4 µl of PCR-grade water instead and (endogenous or exogenous), along with no-template and *B. mallei* positive controls containing DNA of *B. mallei* have to be included in each run to detect amplicon contamination or amplification failure, must be included to ensure reliability.~~

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

~~To date, A positive PCR result in real-time PCR confirms the diagnosis 'of *Burkholderia mallei*' for an isolate and the diagnosis of 'glanders' in clinical cases. It has to be kept in mind, however, samples. The target of these two PCR methods is based on a mobile element in the *B. mallei* *fliP* gene, which distinguishes it from *B. pseudomallei*. It is important to note that future genetic evolution may well result in *B. mallei* clones that can no longer be detected by these standard PCRs, could lead to the loss of this element in *B. mallei* strains, potentially causing false-negative results with *fliP*-based PCR tests (Laroucau *et al.*, 2021). The use of multiple PCR systems is therefore recommended, in complement to serological methods, especially in regions where *B. mallei* strains are poorly characterised (Laroucau *et al.*, 2021).~~

~~The sensitivity of the PCR assays for clinical samples is unknown, so a negative result therefore, is no proof of does not rule out the absence-presence of *B. mallei* in the sample and other diagnostic means must be applied-used for confirmation.~~

2.3.3. PCR assay for *B. pseudomallei* detection

~~No PCR methods have been validated for the detection of *B. pseudomallei* in equine samples, but several PCR systems are available for the specific identification of this species. Among the available targets, the *orf11* PCR (Thibault *et al.*, 2004) (Table 2) is commonly used, for identification or as differential diagnosis with *B. mallei*.~~

2.4. Other methods

2.4. Molecular typing methods and whole genome sequencing

~~Molecular typing techniques for *Burkholderia* isolates such as PCR restriction fragment length polymorphism (Tanpiboonsak *et al.*, 2004), pulsed field gel electrophoresis (Chantratita *et al.*, 2006), ribotyping (Harvey & Minter, 2005), Multi-locus sequence typing (MLST) (Godoy *et al.*, 2003) reveals considerable diversity among *B. pseudomallei* strains, with variation associated with geographical origin.~~

In contrast, *B. mallei* strains are nearly identical worldwide and are characterised by a single sequence type (ST40). Multi-locus or-variable number of tandem repeat analysis (MLVA) has been developed to differentiate *B. mallei* strains (Currie *et al.*, 2009; Singha *et al.*, 2021), are only appropriate for use in but it requires specialised laboratories. Molecular typing laboratory equipment and is mainly used to compare strains from the same outbreak.

More advanced methods such as whole genome sequencing (WGS) and molecular typing techniques such as core genome MLST (cgMLST) (Appelt *et al.*, 2022; Brangsch *et al.*, 2022) and single nucleotide polymorphism (SNP) typing may be useful in the future (Gilling *et al.*, 2014; McRobb *et al.*, 2015; Price *et al.*, 2015; Girault *et al.*, 2018) offer new opportunities to study both *B. mallei* and *B. pseudomallei*. These methods provide the highest resolution and discriminatory power for differentiating isolates and conducting epidemiological studies.

3. Serological tests

Burkholderia mallei evolved from *B. pseudomallei*, which means that infections caused by the two species are currently indistinguishable by serological tests (Neubauer *et al.*, 1997; Wernery *et al.*, 2019b). As a result, serological methods cannot reliably differentiate between the two species, and definitive diagnosis can only be made by direct pathogen identification. WOA Reference Laboratories can provide positive reference sera for serological testing.

3.1. Complement fixation test in horses, donkeys, and mules

The CFT is an accurate a reliable serological test that has been used for many years for diagnosing the diagnosis of glanders and establishing freedom from infection prior to movement. It will deliver positive results detects infections within 1 week post of infection and will also recognise can identify sera from exacerbated chronic cases. Application of rigorous Strict quality control in the formulation of CFT antigens reagents (antigen, complement and haemolytic systems are crucial system) is essential for the performance of this test as its, with both specificity and sensitivity are critically dependent depending on the antigen used (Elschner *et al.*, 2011; Khan *et al.*, 2011). However, the specificity of the CFT has been questioned (Neubauer *et al.*, 2005). The CFT is valid has been validated for horses, mules and camels; if used, but care must be taken when using the test in donkeys particular care is needed to avoid misdiagnosis.

3.1.1. Antigen preparation

- i) The stock culture strain of *B. mallei* stored at -80°C is should be revived by plating onto sheep blood agar and incubated at 37°C for 48 hours to get a confluent growth.
- ii) From this 48 hours culture, a loopful A loop (0.5 mm diameter) of the 48-hour culture is then inoculated into 5 ml of brain-heart infusion (BHI) broth with 3% glycerol and incubated at 37°C for 24 hours.
- iii) After 24 hours, 1 ml from of the above culture broth is further inoculated into 100 ml BHI broth with 3% glycerol and incubated at 37°C for 48 hours with gentle agitation shaking.
- iv) The cultures are inactivated by exposing the flasks to flowing steam (100°C) for 60 minutes.
- v) The clear supernatant is decanted and, filtered: and the filtrate is re-heated again by exposure to live steam for 1 hour, and, It is then clarified by centrifugation at 5000 g for 10 minutes.
- vi) The clarified product supernatant is stored as concentrated antigen in brown glass bottles to shield at 4°C, protected from light and stored at 4°C. Antigen has been shown to be. The antigen is stable for at least 10 years in this concentrated state for at least 10 years.
- vii) Aliquots of antigen are prepared by diluting the concentrated antigen are prepared by dilution 1/20 with sterile physiological saline containing 0.5% phenol. The diluted antigen is dispensed into brown-glass vials and stored at 4°C. The final working dilution is determined by a block titration. The final working dilution for the CFT is prepared when performing the test, and prepared at the time of testing. The resulting antigen consists mainly of lipopolysaccharides (LPS).

The resulting antigen consists primarily of lipopolysaccharides (LPS). An alternative procedure method is to use young cultures by growing the organism, in which *B. mallei* is grown on glycerol-agar slopes for up to 48 hours and washing them off washed with normal saline. A suspension of the culture is heated at 70°C for 1 hour at 70°C and the heat-treated bacterial suspension is used as antigen. The disadvantage of this antigen preparation

method is that the antigen contains all the bacterial cell components. The antigen should be safety tested by ~~inoculating~~ inoculation onto blood agar plates.

Commercial antigens are available (Khan *et al.*, 2011).

3.1.2. CFT procedure

- i) Serum is diluted 1/5 in veronal (barbiturate) buffered saline containing 0.1% gelatine (VBSG), or in CFD (complement fixation diluent – available as tablets) without gelatine or other commercially provided CFT buffers.
- ii) The diluted serum is inactivated at 58–60°C for 30 minutes ~~at 58–60°C~~. Serum of equidae other than horses should be inactivated at 63°C for 30 minutes ~~–, while camel serum is should be~~ inactivated at 56°C for 30 minutes ~~at 56°C~~.
- iii) Twofold dilutions of the sera are prepared ~~using veronal in CFT buffer or alternative commercially available CFT buffers~~ in 96-well round-bottom microtitre plates.
- iv) Guinea-pig complement is diluted in ~~the chosen CFT~~ buffer and 4 or 5 complement haemolytic units-50% (CH₅₀) are used for the analysis.
- v) Sera, complement and antigen are mixed in the plates and incubated for 1 hour at 37°C. An alternative more sensitive procedure is to incubate overnight ~~incubation~~ at 4°C.
- vi) A 2-3% suspension of sensitised washed sheep red blood cells is added to the plates.
- vii) Plates are incubated at 37°C for 45 minutes ~~at 37°C~~, and then centrifuged at 600 g for 5 minutes ~~at 600 g~~.

Note: When using commercially available CFT-antigens and ready-to-use CFT reagents, the manufacturers' instructions should be applied followed.

~~Recommended~~ Controls to verify test conditions:

- i) Positive control: a control serum ~~that known to~~ gives a positive reaction;
- ii) Negative control serum: a control serum ~~that known to~~ gives a negative reaction;
- iii) Anti-complementary control (serum control): ~~diluent, for each serum to be tested~~; CFT buffer + inactivated test serum + complement + haemolytic system;
- iv) Antigen control: ~~diluent CFT buffer~~ + antigen + complement + haemolytic system;
- v) Haemolytic system control: ~~diluent CFT buffer~~ + haemolytic system;
- vi) Complement control: ~~diluent CFT buffer~~ + complement titration + antigen + haemolytic system.

3.1.3. Reading the results

~~The absence of~~ When all test controls (antigen, haemolytic system, complement, negative and positive controls) are valid and no anti-complementary activity must be checked for each is detected in the test serum; anti-complementary sera must be excluded from analyses. A sample that produces, the degree of haemolysis of the test serum can be evaluated. A serum with 100% haemolysis at the 1/5 dilution is negative, 25–75% haemolysis is suspicious-suspect, and no haemolysis (100% fixation) is positive. False-positives results can may occur, and animals can may remain positive for months. Moreover, Animals infected with *B. mallei* and *B. pseudomallei* and *B. mallei* produce cross react and cannot be differentiated-reactive antibodies, making them indistinguishable by serology-CFT (Neubauer *et al.*, 1997). Healthy non-glanders equids; Wernery *et al.*, 2019b). An intradermal mallein test can show a false-produce CFT-positive CFT reaction-results for a variable period of time following a mallein intradermal test.

3.2. Enzyme-linked immunosorbent assays

Both plate and membrane based ELISAs have been used for the serodiagnosis of glanders, but none of these procedures has been able to differentiate between *B. mallei* and *B. pseudomallei*. An avidin-biotin dot ELISA has been described, but has not yet been widely used or validated. The antigen used is a concentrated and purified heat-inactivated bacterial culture. A spot of this antigen is placed on a nitrocellulose dipstick. Using antigen dotted, pre-blocked dipsticks, the test can be completed in approximately 1 hour. An I-ELISA was shown to be of limited value for the serological diagnosis of glanders (Sprague *et al.*, 2009). An I-ELISA based on recombinant *Burkholderia* intracellular motility A protein (rBimA) showed a promising sensitivity of 100% and a specificity of 98.88% (Kumar *et al.*, 2011). Pal *et al.* (2012) used also recombinant proteins to develop an ELISA.

A C-ELISA that makes use of an uncharacterised anti-LPS MAb has also been developed and found to be similar to the CFT in performance (Katz *et al.*, 2000). The C-ELISA was used again on a panel of horse sera originating mainly from Middle Eastern countries (Sprague *et al.*, 2009). A commercially available C-ELISA has recently been developed using anti-s *B. mallei* LPS MAb along with antigen prepared from a regional *B. mallei* isolate. This showed higher sensitivity than CFT in identifying field cases. The C-ELISA has been evaluated on donkey sera and reliable results obtained in an infection trial. Continuing development of monoclonal antibody reagents specific for *B. mallei* antigenic components will offer the possibility to develop more specific ELISAs that will help to resolve questionable test results of quarantined imported horses (Neubauer *et al.*, 1997).

Several in-house and commercial enzyme-linked immunosorbent assays (ELISAs) based on crude or recombinant antigens have been developed for glanders, but systematic validation studies according to Chapter 1.1.6 Validation of diagnostic assays for infectious diseases of terrestrial animals, are lacking. A recent comparative study of five ELISAs using crude suspension or recombinant antigens showed promising results (Elschner *et al.*, 2019). A commercial recombinant double antigen-based ELISA was validated with 400 negative sera from Germany and 370 positive field samples from equidae collected in India and Pakistan (Elschner *et al.*, 2021). This ELISA showed a significantly higher specificity (99.8%) than the CFT (97%) and similar sensitivities (CFT 96.5%, ELISA 98.1%).

The presence of *B. pseudomallei* in the environment may expose equids living in tropical regions where the bacterium is endemic, potentially leading to infection. However, reported cases of melioidosis remain rare. Exposure to the bacterium can induce a detectable serological response, particularly when sensitive assays such as the commercial recombinant double antigen-based ELISA are used. This assay detects *B. mallei* antibodies (Elschner *et al.*, 2021) and also cross-reacts with *B. pseudomallei* antibodies (Desoutter *et al.*, 2024; Gasqué *et al.*, 2024; Laroucau *et al.*, 2025). This validated ELISA is therefore recommended for: i) to confirm clinical cases (in combination with direct diagnostic methods to distinguish between glanders and melioidosis), alongside associated epidemiological investigations; and ii) in the context of international trade, to prevent exposed or infected animals from entering populations and regions that are free from glanders and melioidosis. In areas endemic for melioidosis, where exposure to *B. pseudomallei* may be frequent, apparently healthy equids with a positive ELISA result should undergo follow-up testing and movement restrictions may be warranted. Further studies are needed to clarify the infectious status and epidemiological role of such animals.

More recently, an in-house competitive ELISA for *B. mallei* infection has been developed based on a monoclonal antibody to *B. mallei* lipopolysaccharide (Wernery *et al.*, 2021).

For melioidosis, no although serological techniques tests developed for glanders may cross-react if not specifically designed for glanders, none have been validated for use in veterinary medicine nor are any commercially available to date.

3.3. Immunoblot assays

Immunoblot assays were have been developed for the serodiagnosis of glanders, but further validation was impossible because of the lack of a positive serum control panel (Elschner *et al.*, 2011; Katz *et al.*, 1999). Recently, the development of an This test was recently included in a comparative assays study with the CFT and a commercial recombinant double antigen-based ELISA using 400 glanders negative from Germany and 370 glanders positive field samples from horses, mules, and donkeys (Elschner *et al.*, 2021). The immunoblot using *B. mallei* LPS antigen was reinitiated. The aim was to obtain a was significantly more sensitive test-specific (99.2%) than the CFT in order to retest false positive CFT sera in non-endemic areas (Elschner *et al.*, 2014) (97.0%). Considering the comparable sensitivities of CFT (96.5%) and the immunoblot (97.3%), the immunoblot is a suitable confirmatory test. However, the test is best suited to single samples, as it is time-consuming and requires experienced laboratories. The developed assay test is based on crude antigen preparations of the from *B. mallei* strains (Bogor, Zagreb and Mukteswar, which are also the basis of most CFT antigen formulations. The antigens are and

Bahrain1) separated by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and subsequently transferred to nitrocellulose membranes. Anti-*B. mallei* LPS antibodies in a serum sample reacting to the antigen on the blot strip are visualised by animal using a species-specific (phosphatase) conjugate and the NBT-BCIP (nitro-blue tetrazolium-5-bromo-4-chloro-3-indolyl-phosphate) colour system. The immunoblot test is scored considered positive if the banding a clear scale band pattern of the *B. mallei* LPS ladder within appears in the 20–60 kDa region is clearly visible, suspicious range, suspect if a weak colour reaction is detected observed and negative if no reaction is seen. 171 sera of glanderous horses and mules from Pakistan and Brazil and 305 sera of negative German horses were investigated and all glanders positive and negative animals were diagnosed correctly, however the test has not been fully validated to date. This test is not able to occurs. This test cannot differentiate glanders from melioidosis infection and it has not yet been evaluated for use in donkeys because of the lack of a significant number of positive control sera.

For melioidosis, no serological techniques have yet been validated for use in veterinary medicine.

3.4. Other serological tests

The rose bengal plate agglutination test (RBT) has been described and validated in Russia for the diagnosis of glanders in horses and other susceptible animals; and has been validated in Russia. The test is based on a heat-inactivated bacterial suspension stained with rose bengal, which is used in a plate agglutination test. In a study in Pakistan, the RBT showed a sensitivity of 90% and a specificity of 100% (Naureen *et al.*, 2007). The antigen is a heat inactivated bacterial suspension coloured with Rose Bengal, which is used in a plate agglutination test.

However, the accuracy of other agglutination and precipitin tests this test is unsatisfactory not ideal for control programmes, as horses with chronic glanders and those or in a debilitated condition poor health may give negative or inconclusive results.

For melioidosis, no serological techniques have yet been validated for use in veterinary medicine.

4. Tests for cellular immunity

4.1. The mallein test

The Mallein purified protein derivative (mallein PPD), which is available a commercially, is a available solution of water-soluble protein fractions of prepared from a heat-treated *B. mallei* suspension (see section C below for details of its preparation and availability). Although the test is not generally widely recommended because of animal welfare concerns, however it can may be useful in remote endemic areas where sample transport or proper cooling of samples is not possible. It depends on The test detects hypersensitivity to mallein in infected horses being hypersensitive to mallein. However, advanced clinical cases in horses and acute cases in donkeys and mules may give inconclusive results requiring additional diagnostic methods. In addition, transient seroconversion after mallein injection has been demonstrated in healthy uninfected horses, resulting in false positive CFT results.

The intradermo-palpebral test is the most sensitive, reliable and specific mallein test for detecting infected perissodactyls or odd-toed ungulates, and has largely displaced replaced other methods. The test involves the intradermal injection of 0.1 ml of concentrated mallein PPD is injected intradermally into the lower eyelid and the test. The reaction is read at 24 and 48 hours. A positive reaction is characterised by a marked oedematous swelling of the eyelid, and there may be possibly accompanied by a purulent discharge from the inner canthus or conjunctiva. This is usually accompanied by and a rise in body temperature. With A negative response, there is usually no reaction typically shows minimal or only a little no swelling of the lower lid.

No data are available for on the use of this mallein PPD for in equids with melioidosis.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

There are no vaccines are available for against glanders or melioidosis.

Mallein PPD is available commercially³ from institutes such as the Central Veterinary Control and Research Institute, 06020 Etlik, Ankara, Turkey or the Pasteur Institute, Bucharest, Romania, Calea Giulesti 333, Cod:060269, Sector 6 (aprovizionare@pasteur.ro). The following information outlines the requirements for the production of mallein PPD.

1. Seed management

~~Mallein PPD is produced using three strains of *B. mallei* are employed in the production of mallein PPD, namely: Bogor strain (originating from Indonesia), Mukteswar strain (India) and Zagreb strain (Yugoslavia). The seed material is kept as a stock of freeze-dried cultures. The strains are subcultured on to glycerol agar at 37°C for 1–2 days. For maintaining. To maintain~~ virulence and antigenicity, the strains ~~may can~~ be passaged in guinea-pigs.

2. Production

Dorset-Henley medium, enriched by the addition of trace elements, is used for the production of mallein PPD. The liquid medium is inoculated with a thick saline suspension of *B. mallei*, grown on glycerol agar. The production medium is incubated at 37°C for about 10 weeks. The bacteria are then killed by steaming for 3 hours in a Koch's steriliser. The fluid is then passed through a layer of cotton wool to remove coarse bacterial clumps. The resulting turbid fluid is cleared by membrane filtration, and one part trichloroacetic acid 40 % is immediately added to nine parts culture filtrate. The mixture is allowed to stand overnight during which the light brownish to greyish precipitate settles.

The supernatant is decanted and discarded. The precipitate is centrifuged for 15 minutes at 2500 **g** and the layer of precipitate is washed three or more times in a solution of 5% NaCl, pH 3, until the pH is 2.7. The washed precipitate is dissolved by stirring with a minimum of an alkaline solvent. The fluid is dark brown and has a pH of 6.7. This mallein concentrate is centrifuged again and the supernatant diluted with an equal amount of a glucose buffer solution. The protein content of this product is estimated by the Kjeldahl method and freeze-dried after it has been dispensed into ampoules.

3. In-process control

During the period of incubation, the flasks are inspected regularly for any signs of contamination, and suspicious flasks are discarded. A typical growth of the *B. mallei* cultures comprises turbidity, sedimentation, some surface growth with a tendency towards sinking, and the formation of a conspicuous slightly orange-coloured ring along the margin of the surface of the medium.

4. Batch control

Each batch of mallein PPD is tested for sterility, safety, preservatives, protein content and potency.

Sterility testing is performed according to the European Pharmacopoeia guidelines.

The examination for safety is conducted on five to ten normal healthy horses by applying the intradermo-palpebral test. The resulting swelling should be, at most, barely detectable and transient, without any signs of conjunctival discharge.

Preparations containing phenol as a preservative should not contain more than 0.5% (w/v) phenol. The protein content should be no less than 0.95 mg/ml and not more than 1.05 mg/ml.

Potency testing is performed in guinea-pigs and horses. The animals are sensitised by subcutaneous inoculation with a concentrated suspension of heat-killed *B. mallei* in paraffin oil adjuvant. Cattle can also be used instead of horses. The production batch is bio-assayed against a standard mallein PPD by intradermal injection in 0.1 ml doses in such a way that complete randomisation is obtained.

In guinea-pigs, the different areas of erythema are measured after 24 hours, and in horses the increase in skin thickness is measured with callipers. The results are statistically evaluated, using standard statistical methods for parallel-line assays.

³ ~~Central Veterinary Control and Research Institute, 06020 Etlik, Ankara, Turkey; Pasteur Institute, Bucharest, Romania, Calea Giulesti 333, Cod:060269, Sector 6~~ aprovizionare@pasteur.ro

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NB: There are WOA Reference Laboratories for glanders (please consult the WOA web site: <http://www.woah.org/en/scientific-expertise/reference-laboratories/list-of-laboratories/>).

Please contact the WOA Reference Laboratories for any further information on diagnostic tests, reagents and diagnostic biologicals for glanders

NB: FIRST ADOPTED IN 1991. MOST RECENT UPDATES ADOPTED IN 2018.

Appendix 1: Glanders
Intended purpose of test: population freedom from infection

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
CFT ☒ ☒ ☐	Serum and complement fixing antibodies against <i>B. mallei</i>	Dsp = 96.4 % (CI 95%: 95.6-97.0) Dse = 98.0% (CI 95%: 95.5-99.4)	1. 254 true-positive field serum samples from Pakistan and India 2. 2,959 true-negative field serum samples from Asia, South-America and Europe	See reference	1. High sensitivity of CFT	1. Significantly less specific than e.g. ELISA and Western blot 2. Commercially limited available reagents 3. Complicated non-harmonised testing protocol 4. Experienced staff needed	Elschner <i>et al.</i> (2019)
CFT ☒ ☒ ☐	Serum and complement fixing antibodies against <i>B. mallei</i>	Dsp = 97.0 % (CI 95%: 94.8-98.4) Dse = 96.5% (CI 95%: 94.1-98.1)	1. 370 true-positive field serum samples from Pakistan and India 2. 400 true-negative field serum samples from Germany (officially free from glanders) tested negative in CFT	See reference	1. Comparable sensitivity of CFT and e.g. ELISA and Western blot	1. Significantly less specific than e.g. ELISA and Western blot 2. Commercially limited available reagents 3. Complicated non-harmonised testing protocol 4. Experienced staff needed	Elschner <i>et al.</i> (2021)
^(a) Double-antigen ELISA ☒ ☒ ☒ Equids	Serum and plasma and <i>B. mallei</i> recombinant proteins	Reference tests were Western blot and CFT Dsp = 99.8% (CI 95%: 98.6-100) Dse = 98.1% (CI 95%: 96.1-99.2)	1. 370 true-positive field serum samples (clinical signs and positive CFT or isolation of <i>B. mallei</i> or molecular detection of <i>B. mallei</i> from Pakistan and India) 2. 400 true-negative field serum samples from Germany (officially free from glanders) tested negative in CFT	See reference	1. Significantly more specific than CFT 2. Comparable sensitivity of CFT and the ELISA 3. Commercially available 4. Rapid and simple testing protocol	1. Validation data based on samples from India and Pakistan only 2. Not sufficiently validated for donkeys and mules 3. Analytical specificity determined only with sera from a European country free of glanders and melioidosis 4. Cross reactivity with <i>B. pseudomallei</i>	Elschner <i>et al.</i> (2021) ILC validation report Desoutter <i>et al.</i> (2024) Laroucau <i>et al.</i> (2025)
Mallein skin test ☒ ☐ ☐	Mallein	N/A	N/A	N/A	N/A	1. Low sensitivity and specificity 2. Animal welfare concerns	N/A
Western blotting ☒ ☐ ☐	Serum and antibodies against <i>B. mallei</i>	Dsp = 99.2 % (CI 95%: 97.8-99.8) Dse = 97.3% (CI 95%: 95.1-98.7)	1. 370 true-positive field serum samples from Pakistan and India 2. 400 true-negative field serum samples from Germany (officially free from glanders) tested negative in CFT	See reference	1. Comparable sensitivity of CFT and e.g. ELISA and Western blot	1. Commercially not available 2. Complicated testing protocol 3. Experienced staff needed 4. Cross reaction with <i>B. pseudomallei</i> antibodies	Elschner <i>et al.</i> (2021)

Key: Dsp = diagnostic specificity; Dse = diagnostic sensitivity

^(a)Horse samples only – care needed with interpretation of test on donkey samples.

Appendix 2: Glanders

Intended purpose of test: individual animal freedom from infection prior to movement

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population used to measure accuracy</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages:</u>	<u>References</u>
^(a) CFT ☒ ☒ ☐	<u>Serum and complement fixing antibodies against <i>B. mallei</i></u>	Dsp = 97.0 % (CI 95%: 94.8-98.4) Dse = 96.5% (CI 95%: 94.1-98.1)	1. 370 true-positive field serum samples from Pakistan and India 2. 400 true-negative field serum samples from Germany (officially free from glanders) tested negative in CFT	<u>See reference</u>	1. Comparable sensitivity of CFT and e.g. ELISA and Western blot	1. Significantly less specific than e.g. ELISA and Western blot 2. Commercially limited available reagents 3. Complicated non-harmonised testing protocol 4. Experienced staff needed	<u>Elschner <i>et al.</i> (2021)</u>
^(a) Double-antigen ELISA ☒ ☒ ☒ Equids	<u>Serum and plasma and <i>B. mallei</i> recombinant proteins</u>	Reference tests were Western blot and CFT Dsp = 99.8% (CI 95%: 98.6-100) Dse = 98.1% (CI 95%: 96.1-99.2)	1. 370 true-positive field serum samples (clinical signs and positive CFT or isolation of <i>B. mallei</i> or molecular detection of <i>B. mallei</i> from Pakistan and India) 2. 400 true-negative field serum samples from Germany (officially free from glanders) tested negative in CFT	<u>See reference</u>	1. Significantly more specific than CFT 2. Comparable sensitivity of CFT and the ELISA 3. Commercially available 4. Rapid and simple testing protocol	1. Validation data based on samples from India and Pakistan only 2. Not sufficiently validated for donkeys and mules 3. Analytical specificity determined only with sera from a European country free of glanders and melioidosis 4. Cross reactivity with <i>B. pseudomallei</i>	<u>Elschner <i>et al.</i> (2021)</u> <u>ILC validation report</u> <u>Desoutter <i>et al.</i> (2024)</u> <u>Laroucau <i>et al.</i> (2025)</u>
<u>Mallein skin test</u> ☒ ☐ ☐	<u>Mallein</u>	<u>N/A</u>	<u>N/A</u>	<u>N/A</u>	<u>N/A</u>	1. Low sensitivity and specificity 2. Animal welfare concerns	<u>N/A</u>
<u>Western blotting</u> ☒ ☐ ☐	<u>Serum and antibodies against <i>B. mallei</i></u>	Dsp = 99.2 % (CI 95%: 97.8-99.8) Dse = 97.3% (CI 95%: 95.1-98.7)	1. 370 true-positive field serum samples from Pakistan and India 2. 400 true-negative field serum samples from Germany (officially free from glanders) tested negative in CFT	<u>See reference</u>	1. Comparable sensitivity of CFT and e.g. ELISA and Western blot	1. Commercially not available 2. Complicated testing protocol 3. Experienced staff needed	<u>Elschner <i>et al.</i> (2021)</u>

Key: Dsp = diagnostic specificity; Dse = diagnostic sensitivity

^(a)Horse samples only – care needed with interpretation of test on donkey samples.

Appendix 3: Glanders
Intended purpose of test: contribute to eradication policies

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
CFT ☒ ☒ ☒	Serum and complement fixing antibodies against <i>B. mallei</i>	Dsp = 97.0 % (CI 95%: 94.8-98.4) Dse = 96.5% (CI 95%: 94.1-98.1)	1. 370 true-positive field serum samples from Pakistan and India 2. 400 true-negative field serum samples from Germany (officially free from glanders) tested negative in CFT	See reference	1. Comparable sensitivity of CFT and e.g. ELISA and Western blot	1. Significantly less specific than e.g. ELISA and Western blot 2. Commercially limited available reagents 3. Complicated non-harmonised testing protocol 4. Experienced staff needed	Elschner <i>et al.</i> (2021)
^(a) Double-antigen ELISA ☒ ☒ ☒ Equids	Serum and plasma and <i>B. mallei</i> recombinant proteins	Reference tests were Western blot and CFT Dsp = 99.8% (CI 95%: 98.6-100) Dse = 98.1% (CI 95%: 96.1-99.2)	1. 370 true-positive field serum samples (clinical signs and positive CFT or isolation of <i>B. mallei</i> or molecular detection of <i>B. mallei</i> from Pakistan and India) 2. 400 true-negative field serum samples from Germany (officially free from glanders) tested negative in CFT	See reference	1. Significantly more specific than CFT 2. Comparable sensitivity of CFT and the ELISA 3. Commercially available 4. Rapid and simple testing protocol	1. Validation data based on samples from India and Pakistan only 2. Not sufficiently validated for donkeys and mules 3. Analytical specificity determined only with sera from a European country free of glanders and melioidosis 4. Cross reactivity with <i>B. pseudomallei</i>	Elschner <i>et al.</i> (2021) ILC validation report Desoutter <i>et al.</i> (2024) Laroucau <i>et al.</i> (2025)
Mallein skin test ☒ ☐ ☐	Mallein	N/A	N/A	N/A	N/A	1. Low sensitivity and specificity 2. Animal welfare concerns	N/A
Western blotting ☒ ☒ ☐	Serum and antibodies against <i>B. mallei</i>	Dsp = 99.2 % (CI 95%: 97.8-99.8) Dse = 97.3% (CI 95%: 95.1-98.7)	1. 370 true-positive field serum samples from Pakistan and India 2. 400 true-negative field serum samples from Germany (officially free from glanders) tested negative in CFT	See reference	1. Comparable sensitivity of CFT and e.g. ELISA and Western blot	1. Commercially not available 2. Complicated testing protocol 3. Experienced staff needed	Elschner <i>et al.</i> (2021)

Key: Dsp = diagnostic specificity; Dse = diagnostic sensitivity

^(a)Horse samples only – care needed with interpretation of test on donkey samples.

Appendix 4: Glanders
Intended purpose of test: confirmation of clinical cases

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
Conventional PCR ☒ ☒ ☐	Lesions, tissues, swabs and primers specific for <i>B. mallei</i>	N/A	N/A	See reference	High specificity Rapid for real-time PCR	Intermediate sensitivity Time consuming	Scholz <i>et al.</i> (2006)
Real-time PCR ☒ ☒ ☒	Lesions, tissues, swabs and primers specific for <i>B. mallei</i>	N/A	N/A	See reference	High sensitivity and specificity Rapid	Need expensive lab equipment	Tomaso <i>et al.</i> (2006)
Culture ☒ ☒ ☐	Lesions, tissues, swabs on recommended culture media	N/A	N/A	N/A	Obtaining isolates for further characterisation	Low to intermediate sensitivity	
CFT ☒ ☒ ☐	Serum and complement fixing antibodies against <i>B. mallei</i>	Dsp = 96.4 % (CI 95%: 95.6-97.0) Dse = 98.0% (CI 95%: 95.5-99.4)	1. 254 true-positive field serum samples from Pakistan and India 2. 2,959 true-negative field serum samples from Asia, South-America and Europe	See reference	1. High sensitivity of CFT	1. Significantly less specific than e.g. ELISA and Western blot 2. Commercially limited available reagents 3. Complicated non-harmonised testing protocol 4. experienced staff needed	Elschner <i>et al.</i> (2019)
CFT ☒ ☒ ☐	Serum and complement fixing antibodies against <i>B. mallei</i>	Dsp = 97.0 % (CI 95%: 94.8-98.4) Dse = 96.5% (CI 95%: 94.1-98.1)	1. 370 true-positive field serum samples from Pakistan and India 2. 400 true-negative field serum samples from Germany (officially free from glanders) tested negative in CFT	See reference	1. Comparable sensitivity of CFT and e.g. ELISA and Western blot	1. Significantly less specific than e.g. ELISA and Western blot 2. Commercially limited available reagents 3. Complicated non-harmonised testing protocol 4. experienced staff needed	Elschner <i>et al.</i> (2021)
^(a) Double-antigen ELISA ☒ ☒ ☒ Equids	Serum and plasma and <i>B. mallei</i> recombinant proteins	Reference tests were Western blot and CFT Dsp = 99.8% (CI 95%: 98.6-100) Dse = 98.1% (CI 95%: 96.1-99.2)	1. 370 true-positive field serum samples (clinical signs and positive CFT or isolation of <i>B. mallei</i> or molecular detection of <i>B. mallei</i> from Pakistan and India) 2. 400 true-negative field serum samples from Germany (officially free from glanders) tested negative in CFT	See reference	1. Significantly more specific than CFT 2. Comparable sensitivity of CFT and the ELISA 3. Commercially available 4. Rapid and simple testing protocol	1. Validation data based on samples from India and Pakistan only 2. Not sufficiently validated for donkeys and mules 3. Analytical specificity determined only with sera from a European country free of glanders and melioidosis 4. Cross reactivity with <i>B. pseudomallei</i>	Elschner <i>et al.</i> (2021) ILC validation report Desoutter <i>et al.</i> (2024) Laroucau <i>et al.</i> (2025)

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Mallein skin test</u> ☒ ☐ ☐	<u>Mallein</u>	<u>N/A</u>	<u>N/A</u>	<u>N/A</u>	<u>N/A</u>	1. <u>Low sensitivity and specificity</u> 2. <u>Animal welfare concerns</u>	<u>N/A</u>
<u>Western blotting</u> ☒ ☐ ☐	<u>Serum and antibodies against <i>B. mallei</i></u>	<u>Dsp = 99.2 % (CI 95%: 97.8-99.8)</u> <u>Dse = 97.3% (CI 95%: 95.1-98.7)</u>	1. <u>370 true-positive field serum samples from Pakistan and India</u> 2. <u>400 true-negative field serum samples from Germany (officially free from glanders) tested negative in CFT</u>	<u>See reference</u>	1. <u>Comparable sensitivity of CFT and e.g. ELISA and Western blot</u>	1. <u>Commercially not available</u> 2. <u>Complicated testing protocol</u> 3. <u>Experienced staff needed</u>	<u>Elschner <i>et al.</i> (2021)</u>

Key: Dsp = diagnostic specificity; Dse = diagnostic sensitivity

^(a)Horse samples only – care needed with interpretation of test on donkey samples.

Appendix 5: Glanders
Intended purpose of test: prevalence of infection – surveillance

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
CFT ☒ ☒ ☒	Serum and complement fixing antibodies against <i>B. mallei</i>	Dsp = 96.4 % (CI 95%: 95.6-97.0) Dse = 98.0% (CI 95%: 95.5-99.4)	1. 254 true-positive field serum samples from Pakistan and India 2. 2,959 true-negative field serum samples from Asia, South-America and Europe	See reference	1. High sensitivity of CFT	1. Significantly less specific than e.g. ELISA and Western blot 2. Commercially limited available reagents 3. Complicated non-harmonised testing protocol 4. Experienced staff needed	Elschner <i>et al.</i> (2019)
CFT ☒ ☒ ☒	Serum and complement fixing antibodies against <i>B. mallei</i>	Dsp = 97.0 % (CI 95%: 94.8-98.4) Dse = 96.5% (CI 95%: 94.1-98.1)	1. 370 true-positive field serum samples from Pakistan and India 2. 400 true-negative field serum samples from Germany (officially free from glanders) tested negative in CFT	See reference	1. Comparable sensitivity of CFT and e.g. ELISA and Western blot	1. Significantly less specific than e.g. ELISA and Western blot 2. Commercially limited available reagents 3. Complicated non-harmonised testing protocol 4. Experienced staff needed	Elschner <i>et al.</i> (2021)
^(a) Double-antigen ELISA ☒ ☒ ☒ Equids	Serum and plasma and <i>B. mallei</i> recombinant proteins	Reference tests were Western blot and CFT Dsp = 99.8% (CI 95%: 98.6-100) Dse = 98.1% (CI 95%: 96.1-99.2)	1. 370 true-positive field serum samples (clinical signs and positive CFT or isolation of <i>B. mallei</i> or molecular detection of <i>B. mallei</i> from Pakistan and India) 2. 400 true-negative field serum samples from Germany (officially free from glanders) tested negative in CFT	See reference	1. Significantly more specific than CFT 2. Comparable sensitivity of CFT and the ELISA 3. Commercially available 4. Rapid and simple testing protocol	1. Validation data based on samples from India and Pakistan only 2. Not sufficiently validated for donkeys and mules 3. Analytical specificity determined only with sera from a European country free of glanders and melioidosis 4. Cross reactivity with <i>B. pseudomallei</i>	Elschner <i>et al.</i> (2021) ILC validation report Desoutter <i>et al.</i> (2024) Laroucau <i>et al.</i> (2025)
Mallein skin test ☒ ☐ ☐	Mallein	N/A	N/A	N/A	N/A	1. Low sensitivity and specificity 2. Animal welfare concerns	N/A
Western blotting ☒ ☒ ☐	Serum and antibodies against <i>B. mallei</i>	Dsp = 99.2 % (CI 95%: 97.8-99.8) Dse = 97.3% (CI 95%: 95.1-98.7)	1. 370 true-positive field serum samples from Pakistan and India 2. 400 true-negative field serum samples from Germany (officially free from glanders) tested negative in CFT	See reference	1. Comparable sensitivity of CFT and e.g. ELISA and Western blot	1. Commercially not available 2. Complicated testing protocol 3. Experienced staff needed	Elschner <i>et al.</i> (2021)

Key: Dsp = diagnostic specificity; Dse = diagnostic sensitivity

^(a)Horse samples only – care needed with interpretation of test on donkey samples.

MEETING OF THE BIOLOGICAL STANDARDS COMMISSION

Paris, 8–12 September 2025

CHAPTER 3.8.3.

CONTAGIOUS AGALACTIA

SUMMARY

Description and importance of the disease: Contagious agalactia is a serious disease syndrome of sheep and goats the primary target of which is the udder leading to an interstitial mastitis that results in losses in milk production and changes to its composition ~~that is characterised by mastitis, Arthritis, keratoconjunctivitis and, occasionally, abortion are secondary manifestations of the disease.~~ Mycoplasma agalactiae (Ma) is the historical and sole main cause of the classical form of the disease in domestic sheep and goats, although other mycoplasmas, including the respiratory and articular pathogens ~~but M. capricolum subsp. capricolum (Mcc), M. mycoides subsp. capri (Mmc), M. capricolum subsp. capricolum (Mcc) and M. putrefaciens (Mp) can produce a clinically similar disease, more often in but only in goats, which may be accompanied by pneumonia.~~ Ma and Mcc have been isolated from wild small ruminants such as ibex and mountain goats, which may act as a reservoir of infection for domestic species. ~~Antibodies to Mmc and Mcc have been detected in South American camelids (alpacas, llamas and vicunas), but no mycoplasmas have yet been isolated.~~

Identification of the agent: Definitive diagnosis requires the isolation of the causative mycoplasmas from the affected animals, which are then identified by biochemical, serological and, ~~increasingly,~~ molecular tests such as the polymerase chain reaction (PCR). Samples of choice include milk, conjunctival and ear swabs, and joint fluid. The sampling of bulk milk tank provides a convenient way of monitoring whole herds/flocks for causative mycoplasmas. The causative mycoplasmas grows relatively well in most mycoplasma media although Ma ~~agalactiae~~ shows a preference for organic acids such as pyruvate as substrates.

Serological tests: Detection of antibodies in serum by enzyme-linked immunosorbent assay (ELISA) provides rapid diagnosis of disease but may not be very sensitive in chronically affected herds and flocks. Indirect ELISAs have been used routinely in control programmes for screening herds for Ma. Confirmation of infection by isolation and identification or detection by ~~polymerase chain reaction~~ PCR is usually necessary in areas believed to be free of contagious agalactia. ~~Serological tests are not widely available for M. putrefaciens.~~

Requirements for vaccines: Commercial vaccines for Ma, inactivated with formalin, are widely used in southern Europe, but are not considered to be very efficacious. Under experimental conditions, Ma vaccines inactivated with saponin have been shown to be more protective than formalised preparations. Live vaccines for Ma are used in Türkiye, where they are reported to be more protective than inactivated vaccines. A commercial vaccine containing Ma, Mmc and Mcc is available. ~~Autogenous vaccines for Mmc and, occasionally, for Mcc are believed to be used in some countries. No vaccines exist for M. putrefaciens, as the disease it causes is not considered to be sufficiently serious or widespread.~~

A. INTRODUCTION

Contagious agalactia (CA) is a disease of sheep and goats that is characterised by mastitis, arthritis and keratoconjunctivitis, and has been known for nearly 200 years. The disease is found wherever sheep and goats are kept, but is mainly concentrated in countries where pastoralism and small ruminant dairy production is located in Europe, particularly in surrounding the Mediterranean regions, and western Asia, North Africa, and sporadically in the United States of America (USA) where pastoralism and small ruminant dairy production is practised. It is difficult to estimate the full scale of the problem as few countries have carried out prevalence studies. It is mainly caused by *Mycoplasma agalactiae* (Ma) (Bergonier *et al.*, 1997). An upsurge of contagious agalactia, caused by Ma, has been seen in sheep in Spain and France recently with increased cases reported in and around the Pyrenees and new outbreaks being reported in Corsica (Chazel *et al.*, 2010). Italy still reports more than 50 outbreaks every year, mainly notified on two of its Islands¹. Frequent and numerous outbreaks occur in Iran and Mongolia (Nicholas *et al.*, 2008). *Mycoplasma capricolum* subsp. *capricolum* (Mcc) and *M. mycoides* subsp. *capri* (formerly *M. mycoides* subsp. *mycoides* LC [LC = large colonies]) have also been isolated from sheep and goats with mastitis and arthritis in many countries including Italy, where the disease is spreading (Marogna *et al.*, 2015), South America (Nascimento *et al.*, 1986) and Australasia (Cottow, 1971). Data compiled by WOAHA through its global animal health reference database (WAHIS) provides some indication, although not all countries report the number of outbreaks they have (Jaÿ & Tardy, 2019), probably because they do not have mycoplasma diagnostic laboratories, while others, simply declare the disease is present (WAHIS 2025, accessed 30/04/2025). These figures probably represent an underestimate because of the difficulty in detecting infected flocks and herds, many of which may not show overt clinical signs, along with the reluctance of farmers/veterinarians to report outbreaks (Loria *et al.*, 2019).

The clinical signs of infections caused by Mcc, Mmc and *M. putrefaciens* (Mp) are sufficiently similar to be considered indistinguishable from contagious agalactia caused by Ma. In addition, Mp also causes mastitis and arthritis in goats, which is very similar to that caused by Ma, Mmc and Mcc (Rodriguez *et al.*, 1994). Furthermore, the consensus of the working group on contagious agalactia of the EC COST² Action 826 on ruminant mycoplasmoses, which met in Toulouse, France, in 1999, was that all four mycoplasmas should be considered as causal agents of contagious agalactia. In France, Mmc, Mcc and Mp constitute over 80% of the mycoplasma isolations from goats with Ma accounting for less than 2%. Ma causes CA in both sheep and goats, but Mcc have also been isolated from wild small ruminants such as ibex and mountain goats in the Pyrenees and Alps (Chazel *et al.*, 2010; Verbsiek *et al.*, 2008). There are occasional reports of the isolation of Ma (Chazel *et al.*, 2010) and Mcc from apparently healthy cattle (Pinho *et al.*, 2009), with Catania *et al.* (2016) isolating Ma from eyes, ears and brain spinal fluid of bulls.

Clinically, the disease caused by Ma is recognised by elevated temperature, inappetence and alteration in the consistency of the milk in lactating ewes with decline and subsequent failure of milk production, often within 2–3 days, as a result of CA caused by *Mycoplasma agalactiae* (Ma) is typically first noticed when milk production falls, usually a few days after the introduction of healthy carriers, or after mixing with affected animals at pasture, markets or water sources. The primary target of Ma is the udder, where it causes interstitial mastitis leading to raised temperature and inappetence. Milk becomes abnormal in appearance, mastitis develops and then milk production reduces, or ceases, in one or both halves of the udder, occasionally permanently. (Bergonier *et al.*, 1997); lameness and Keratoconjunctivitis and arthritis are chronic sequelae, which may be particularly severe in affects about 5–10% of infected young animals, which prevents them keeping up with the group during transhumance and other animal movements. Fever is common in acute cases and may be accompanied by nervous signs, but these are rare in the more frequently observed subacute and chronic infections where the disease is endemic. Pregnant animals may abort. Ma may occasionally be found in lung lesions (Loria *et al.*, 1999), but respiratory disease is rarely a feature of CA caused by Ma and, where it has been reported, may have been the result of a mixed infection with other pathogens, in particular *Mycoplasma mycoides* subspecies *capri* (Mmc) (Jaÿ & Tardy, 2019) pneumonia is not a consistent finding. Bacteraemia is common, particularly for Mmc and Mcc and could account for the isolation of the organism from sites where it is only present transiently.

Over the past 50 years, three other mycoplasmas – Mmc, *Mycoplasma capricolum* subsp. *capricolum* (Mcc) and *Mycoplasma putrefaciens* (Mp) belonging to a phylogenetically distinct group (Manso-Silvan *et al.*, 2007), have been added to the aetiology of CA because they can cause similar clinical signs, although almost exclusively in goats (Migliore *et al.*, 2021). Arthritis, pleurisy, pneumonia, mastitis and keratoconjunctivitis may all result from infection with However, while Mmc, has one of the widest geographical distributions of ruminant mycoplasmas, being found on all continents where small ruminants are kept is widely distributed geographically and underestimated as a respiratory pathogen, the disease it causes is in many cases a clinically distinct from that caused by Ma (Migliore *et al.*, 2021), with the pathogen displaying a pronounced respiratory localisation and wherever contagious agalactia and caprine pleuropneumonia are reported (DaMassa *et al.*, 1983; Nicholas, 2002); however the lack of diagnostic facilities for mycoplasma diseases in many countries means that it is probably under reported. Mmc is mostly confined to goats but has occasionally been isolated from sheep with reproductive disease and cattle with arthritis or respiratory disease. Cases usually occur sporadically and the disease may persist and

1 — Data from WOAHA World Animal Health Information System: <https://wahis.woah.org/#!/home>

2 — European Cooperation in the field of Scientific and Technical Research.

spread slowly within a herd; nevertheless, in free areas, the disease shows high morbidity and mortality of kids. In Sicily a reported outbreak showed more than 40% mortality (Agnello *et al.*, 2012). After parturition, the opportunity for spread in milking animals increases, and kids ingesting infected colostrum and milk become infected. The resulting septicaemia, with arthritis and pneumonia, causes high mortality in kids (Bergonier *et al.*, 1997). ~~DaMassa *et al.*, 1983). *Mcc* also favours the respiratory and articular systems, is widely distributed and highly pathogenic, particularly in North Africa, but the frequency of occurrence is low is less commonly reported, particularly outside France and Morocco (Bergonier *et al.*, 1997). However, in some CA endemic regions *Mmc* (and occasionally *Mcc*) can, like *Ma*, persist in goat herds with a prior history of infection, with intermittent shedding in milk of apparently healthy lactating goats (de la Fe *et al.*, 2025; Tardy *et al.*, 2019). Goats are more commonly affected than sheep, and clinical signs of fever, septicaemia, mastitis, and severe arthritis may be followed rapidly by death (Bergonier *et al.*, 1997; Bolske *et al.*, 1988). Pneumonia may be seen at necropsy. The severe joint lesions seen in experimental infections with this organism are accompanied by intense periarticular subcutaneous oedema affecting tissues some distance from the joint (Bolske *et al.*, 1988). In 2015 the first human isolate of *Mcc* was reported from a man with septicaemia (Seersholm *et al.*, 2015).~~

Mp is a very infrequent isolate of marginal pathogenicity, often recovered from apparently healthy goats and found occasionally with *Ma* where it plays very little role in disease progression (Gil *et al.*, 2003; Hajizadeh *et al.*, 2018). ~~common in milking goat herds in western France where it can be isolated from animals with and without clinical signs (Mercier *et al.*, 2004). It has also been associated with a large outbreak of mastitis and agalactia leading to severe arthritis in goats accompanied by abortion and death without pyrexia in California, USA (Bergonier *et al.*, 1997). *Mp* was the major finding in an outbreak of polyarthritis in kids in Spain (Rodriguez *et al.*, 1994).~~

Antibodies to *Mmc* and *Mcc*, but not *Ma*, have been detected in South American camelids, including llamas, alpacas and vicunas, but as yet no mycoplasmas have been isolated (Nicholas, 1998). These camelids are affected by a range of mycoplasma-like diseases, including polyarthritis and pneumonia, so it is likely that mycoplasmas including *Mmc* and *Mcc* may be found in the future.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of contagious agalactia and their purpose

Method	Purpose					
	Population freedom from infection ^(a)	Individual animal freedom from infection prior to movement ^(a)	Contribute to eradication policies ^(a)	Confirmation of clinical cases ^(a)	Prevalence of infection – surveillance ^(a)	Immune status in individual animals or populations post-vaccination ^(a)
Identification of the agent ^(g)						
Culture and identification of the organism	☑☑☐	☑☑☑	☑☑☑	☑☑☑	☐☐☐-☑☐☐	☐☐☐
Conventional PCR	☑☑☐	☑☑☑	☐☐☐	☑☑☑	☑☑☐	☐☐☐
Real-time PCR	☑☑☐	☑☑☑	☐☐☐-☑☐☐	☑☑☑	☑☑☐	☐☐☐
<u>LAMP</u>	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☑☑☐	☐☐☐
Detection of immune response						
CFT	☑☑☑*	☑☐☐	☑☑☑	☑☑☑*	☑☑☑*	☑☑☑*
ELISA	☑☑☑**	☑☐☐	☑☑☑	☑☑☑**	☑☑☑**	☑☑☑**
Immunoblotting	☑☐☐	☑☑☐	☑☐☐	☑☑☐	☑☐☐	☑☑☐

Key: ☑☑☑ = recommended for this purpose; ☑☑☐ = recommended but has limitations;

☑☐☐ = suitable in very limited circumstances; ☐☐☐ = not appropriate for this purpose.

*for *Mcc* and *Mmc* only; **for *Ma* only.

PCR = polymerase chain reaction; LAMP: loop-mediated isothermal amplification;

CFT = complement fixation test; ELISA = enzyme-linked immunosorbent assay.

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

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

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

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

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

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

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

1. Identification of the agents

1.1. Selection of ~~specimens~~ samples

Preferred ~~samples~~ specimens from living animals include: milk from mastitic females or from apparently healthy females; nasal swabs and secretions; where there is a high rate of mortality/morbidity in kids; joint fluid from arthritic cases; conjunctival swabs from cases of ocular disease; and blood for antibody detection from affected and non-affected animals (Nicholas & Baker, 1998). The sampling of bulk milk tank provides a convenient way of monitoring flocks and herds for causative mycoplasmas (de la Fe *et al.*, 2025; Tardy *et al.*, 2019). ~~A distinctive smell of putrefaction in the milk is often the first sign of the presence of *Ma* infection in a herd.~~ The ear canal has also been shown to be a source of pathogenic mycoplasmas, although in practice the presence of non-pathogenic mycoplasmas at this site may make confirmation difficult (Nicholas & Baker, 1998). Mycoplasmas may be isolated from the blood during the acute stage of the disease when there is mycoplasmaemia. From dead animals, ~~samples~~ specimens should include: udder and associated lymph nodes, joint fluid, lung tissue (at the interface between diseased and healthy tissue) and pleural/pericardial fluid. ~~Samples~~ Specimens should be dispatched quickly to a diagnostic laboratory in a moist and cool condition. ~~All four~~ Causative mycoplasmas are relatively easy to isolate from internal organs, joints and milk and grow well in most mycoplasma media, producing medium to large colonies in 3–4 days.

1.2. *Mycoplasma* isolation

The usual techniques used in the isolation of mycoplasmas apply ~~to all four causative organisms~~ (Nicholas & Baker, 1998). Many media have been reported to grow the causative mycoplasmas. Improved growth rates of *Ma* have been seen in media containing organic acids such as pyruvate and isopropanol (Khan *et al.*, 2004). The formulation of PRM medium (Khan *et al.*, 2004) is as follows:

Heat-inactivated porcine serum 100 ml/litre, special peptone 20 g/litre, yeast extract 5 g/litre, glycerol 5 g/litre, sodium chloride 5 g/litre, HEPES (N-2-hydroxyethylpiperazine, N-2-ethanesulphonic acid) 9 g/litre, fresh yeast extract 100 ml/litre, sodium pyruvate 5 g/litre, 12.5 ml of 0.2% phenol red and ampicillin (200,000 International Units/ml. Make up to 1 litre in distilled water and sterilise by filtration. Adjust the pH of the broth medium to 7.6. Prepare solid medium by adding 10 g of LabM agar No. 1 (Bury UK, or agar of equivalent quality) and dispense into sterile Petri dishes. Pleuropneumonia-like organisms (PPLO) (Mycoplasma) agar bases with associated supplements are widely available commercially.

Thallium acetate (250 mg/litre), which is toxic and inhibitory to some mycoplasmas but not those causing ~~contagious agalactia-CA, or colonising the same body site~~, may be a necessary component of the transport medium to reduce general bacterial contamination from clinical samples, but should be omitted once the mycoplasmas begin to grow *in vitro*. A satisfactory alternative to thallium acetate may be colistine sulphate (37.5 mg/litre).

1.2.1. Test procedure

- i) Make tenfold dilutions (10^1 – 10^6) of the liquid sample (milk, synovial fluid, conjunctival and ear swabs) or tissue homogenate in appropriate broth medium.
- ii) Spread a few drops of each sample on the agar medium and dispense a 10% (v/v) inoculum into broth medium.
- iii) Streak swabs directly on to agar medium.
- iv) Incubate inoculated broths (optimally with gentle shaking) and agar media at 37°C in humidified atmosphere with 5% carbon dioxide.
- v) Examine broths daily for signs of growth (indicated by a fine cloudiness or opalescence) or changes in pH indicated by a colour change and examine agar media under $\times 35$ magnification for typical 'fried egg' colonies.
- vi) If no mycoplasma growth is seen after 7 days, subculture a 10% (v/v) inoculum of broth into fresh broth and spread about 50 μ l of this on to agar media.

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- vii) Repeat as for step v. If no mycoplasmas are seen after 21 days' incubation, consider the results to be negative.
 - viii) If bacterial contamination results (seen as excessive turbidity), filter sterilise by passing 1 ml of contaminated broth through a 0.45 µm filter into fresh broth medium.

Clinical samples frequently contain more than one mycoplasma species so clone purification of colonies is often considered necessary before performing biochemical and serological identification, in particular the growth and film inhibition tests (GIT and FIT, respectively). However, cloning is a lengthy procedure taking at least 2 weeks. The immunofluorescence test (Bradbury, 1998), dot immunobinding tests (Poumarat, 1998) and, more recently, polymerase chain reaction (PCR) tests (see Section B.1.5) do not require cloning as these tests can detect the pathogenic mycoplasmas in mixed cultures, saving a great deal of time.

1.3. Biochemical tests

The first test that should be performed on the cloned isolates is sensitivity to digitonin, which separates mycoplasmas from acholeplasmas; the latter are ubiquitous contaminants that can overgrow the mycoplasmas of interest. Growth in liquid medium containing glucose (1%), arginine (0.2%), and phenolphthalein diphosphate (0.01%), on solid medium containing horse serum or egg yolk for the demonstration of film and spots, and on casein agar or coagulated serum agar to test for proteolysis, ~~are among previously considered to be the most~~ the most useful tests for differentiating ~~the four *Ma* from the other small ruminant mycoplasmas (Poveda, 1998). *Ma* will produce film and spots on the surface of the broth and solid media. However, these biochemical characteristics, however have been increasingly found to be variable for the individual mycoplasmas and have little diagnostic value. The most impressive biochemical characteristic that differentiates *Mp* from all other mycoplasmas is the produces an odour of putrefaction it produces in broth culture when present in a sample. Other features that may be helpful include: film and spot production seen on the surface of the broth and solid media caused by *Ma* and to a lesser extent by *Mp*; and the proteolytic activity of *Mcc* and *Mmm*LC on casein and coagulated serum.~~

1.4. Serological identification

Identification of isolates using specific antisera is usually carried out with the GIT, FIT (Poveda & Nicholas, 1998) or the indirect fluorescent antibody (IFA) test (Bradbury, 1998). A dot immunobinding test that is carried out in microtitre plates offers many improvements over the other serological tests such as rapidity and higher throughputs (Poumarat, 1998) but requires subjective judgements of staining intensity. For *Ma*, film inhibition may often be more reliable as growth inhibition is not seen with all isolates; it can also be used for serodiagnosis. Film production by the mycoplasma may be enhanced by the incorporation of 10% egg yolk suspension into the solid medium.

1.4.1. Test procedure

- i) Inoculate at least two dilutions of 48-hour cloned broth cultures (10^{-1} and 10^{-2}) on to predried agar media by allowing 50 µl of the cultures to run down the tilted plates using the 'running drop' technique (Poveda & Nicholas, 1998). Remove any excess liquid with a pipette.
- ii) Allow the plates to dry. It is possible to apply two or three well separated running drops to each 90 mm diameter plate.
- iii) Apply pre-dried filter paper discs containing 30 µl of specific antiserum to the culture; ensure good separation of discs (at least 30 mm).
- iv) Incubate the plates as for mycoplasma culture and examine daily by eye against a light background.

1.4.2. Interpretation of the results

A zone of inhibition over 2 mm, measured from the paper disc to the edge of mycoplasma growth is considered to be significant. Partial inhibition can occur with weak antiserum or where there are mixed cultures. Stronger reactions can be obtained if about 60 µl of antiserum is added to 6 mm diameter wells made in the agar with a cork borer or similar device (Poveda & Nicholas, 1998).

In the IFA test, specific antisera are applied to colonies on solid medium. Homologous antiserum remains attached after washing and is demonstrated by adding fluorescein-conjugated antiglobulin, washing, and viewing the colonies with an epifluorescence microscope (Bradbury, 1998). Controls should include known positive and known negative control organisms, and a negative control serum. However like the immunobinding tests subjective judgements are required to assess staining intensity.

Antisera for these serological tests have traditionally been prepared against the type strains of the various *Mycoplasma* species, and most field isolates have been readily identified using these antisera. As more strains have been examined, however, some have been found to react poorly with these antisera, while reacting well with antisera to other representative strains of the species. Intraspecies variation in antigenic composition ~~has not been reported for *Mp*, but occurs to some degree with *Ma* and with *Mcc* strains.~~ Thus, diagnostic laboratories may need to have several antisera to enable all strains of the species to be identified.

1.5. Molecular methods – detection of nucleic acids ~~Nucleic acid recognition methods~~

Extraction of nucleic acids

Different kits and procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid is important and can be checked using a suitable method as appropriate to the circumstances. For molecular testing of DNA extracted directly from the clinical sample, it is important to validate the performance of chosen extraction methods for the relevant sample types.

Table 2. Conventional PCRs

Pathogen/ target gene	Primer/probe (5'–3')	Concentration	Cycling parameters ^(a)
<u>Method 1: Subrahmaniam <i>et al.</i>, 1998; GenBank Accession No. AF003960; amplicon size 1624 bp</u>			
<u><i>Ma/UvrC</i></u>	<u>MAGAUVRC1-L: CTC-AAA-AAT-ACA-TCA-ACA-AGC</u> <u>MAGAUVRC1-R: CTT-CAA-CTG-ATG-CAT-CAT-AA</u>	<u>400 nM</u>	<u>35 cycles of: 94°C/30 sec,</u> <u>50°C/30 sec, 72°C/60 sec</u>
<u>Method 2: Tola <i>et al.</i>, 1997a; GenBank Accession No.: X95628; amplicon size 375 bp</u>			
<u><i>Ma/P80</i></u>	<u>FS1: AAA-GGT-GCT-TGA-GAA-ATG-GC</u> <u>FS2: GTT-GCA-GAA-GAA-AGT-CCA-ATC-A</u>	<u>100 nM</u>	<u>30 cycles of: 94°C/60 sec,</u> <u>57°C/60 sec, 65°C/60 sec</u>
<u>Method 3: Marenda <i>et al.</i>, 2005; GenBank Accession No.: CL844514 to CL844551; amplicon size 265 bp</u>			
<u><i>Ma/PolC</i></u>	<u>MAPol-1F CAT-TGA-ACC-TCT-TAT-GTC-ATT-TAC-TTT-G</u> <u>MAPol-5R CIA-TGT-CAT-CAG-CTT-TTG-GGT-GA</u>	<u>400 nM</u>	<u>30 cycles of: 94°C/30 sec,</u> <u>49°C/30 sec, 72°C/30 sec</u>
<u>Method 4: Manso-Silvan <i>et al.</i>, 2007; GenBank Accession No.: BX293980 and CP000123; amplicon size 781 bp (sequence 582 bp)^(b)</u>			
<u><i>M. mycoides</i></u> <u>cluster –</u> <u>differentiation from</u> <u><i>Ma/FusA</i></u>	<u>FusA-F: TGA-AAT-TTT-TAG-ATG-GTG-GAG-AA</u> <u>FusA-R: GGT-AAT-TTA-ATA-GTT-TCA-CGA-TAT-GAA</u>	<u>400 nM</u>	<u>35 cycles of: 94°C/30 sec,</u> <u>52°C/30 sec, 72°C/45 sec</u>
<u>Method 5: Greco <i>et al.</i>, 2001; amplicon sizes 357 (<i>Ma</i>) 257 (<i>Mmc</i>)/260 (<i>Mcc</i>)^(c)bp</u>			
<u>Simultaneous</u> <u>identification of</u> <u><i>Ma/Mmc/Mcc</i></u>	<u>Ma1: AAA-GGT-GCT-TGA-GAA-ATG-GC</u> <u>Ma2: GTT-GCA-GAA-GAA-AGT-CCA-ATC-A</u> <u>CL1: TAT-ATG-GAG-TAA-AAA-GAC</u> <u>CL2: GCA-TCA-TAA-ATA-ATT-G</u>	<u>100 nM</u>	<u>30 cycles of: 94°C/60 sec,</u> <u>46°C/60 sec, 72°C/60 sec</u>
<u>Method 6: Cillara <i>et al.</i>, 2015; GenBank Accession No.: BX293980 and CP000123; amplicon size 911 bp^(c)/362 and/or 281</u>			
<u>Differentiation</u> <u><i>Mmc</i> from <i>Mcc</i></u> <u>(and from</u> <u><i>Ma</i>)/<i>lpdA</i></u>	<u>LPD-C1-F: AGGTGAAGCTGTTGCTTTAG</u> <u>LPD-C1-R: TTCCAATAATATGTGCACCTAA</u> <u>LPD-F: CGATGGAAAAGATCAAATGG</u> <u>LPD-R: TTGTTGTTTCAGTTTTCCT</u>	<u>Not provided</u>	<u>30 cycles of: 95 °C/60 sec,</u> <u>56 °C/60 sec, 72 °C/90 sec</u> <u>30 cycles of: 95 °C/60 sec,</u> <u>50 °C/60 sec, 72 °C/60 sec</u>
<u>Method 7: Woubit <i>et al.</i>, 2007; GenBank Accession No.: CP000123; amplicon size 375 bp^(c)</u>			
<u><i>M. mycoides</i></u> <u>cluster –</u> <u>differentiation from</u> <u><i>Ma</i></u>	<u>gik-myc-2F: TGC-ACT-TGG-TGA-ATA-TAR-AAA-AGG</u> <u>gik-myc-2R: GGA-TCT-AAA-GCR-TGT-ATT-ART-AAT-G</u>	<u>400 nM</u>	<u>30 cycles of: 94°C/15 sec,</u> <u>50°C/15 sec, 72°C/15 sec</u>
<u>Method 8: Peyraud <i>et al.</i>, 2003; GenBank Accession No.: AY282502; amplicon size 316 bp</u>			

<u>Mp/ArcB</u>	Mput1: AAA-TTG-TTG-AAA-AAT-TAG-CGC-GAC Mput2: CAT-ATC-ATC-AAC-TAG-ATT-AAT-AGT-AGC-ACC	400 nM	30 cycles of: 94°C for 30 sec, 40°C for 30 sec and 72°C for 30 sec
Method 9: McAuliffe <i>et al.</i> , 2005; amplicon size 340 bp			
<u>Differentiates <i>Ma</i> from <i>Mmc</i>, <i>Mcc</i>, <i>Mp/16S rRNA gene</i></u>	GC-341F: CGC-CCG-CCG-CGC-GCG-GCG-GGC-GGG- GCG-GGG-GCA-CGG-GGG-GCC-TAC-GGG-AGG-CAG-CAG R543 ACC-TAT-GTA-TTA-CCG-CG	200 nM	30 cycles of: 95°C/60 sec, 55°C/45 sec, 72°C/60 sec

^(a)A denaturation step prior to cycling, or final extension step, has not been included. ^(b)Sequencing of product is required.

^(c)Digestion or sequencing required.

Table 3. Real-time PCRs

<u>Pathogen/ target gene</u>	<u>Primer (5'–3')</u>	<u>Concentration</u>	<u>Cycling parameters^(a)</u>
Method 1: Lorusso <i>et al.</i> , 2007; GenBank Accession No. X95628; amplicon size: 117 bp			
<u>Ma/P81</u>	Ma For: AAA-CTT-TGA-AGA-TAA-TGA-CAA-A Ma Rev: TGG-AAT-TAT-GAA-TGA-ACC-ATT Probe: 6-FAM-TTA-ATC-TTG-ATA-ATA-TAA-GTG-AAC- AGT-TTA-CTA-CT-BHQ1	600 nM 200 nM	40 cycles of: 95°C/60 sec, 50°C/30 sec and 60°C/60 sec
Method 2: Oravcova <i>et al.</i> , 2009; GenBank Accession No. AJ344229 amplicon size: 109 bp			
<u>Ma/P40</u>	MAP40127F: TCA-TTT-ACA-GCA-GTG-CCT-TTA-TTA-G MAP40235R: CACCTAATGCTTGTTCATCAACC Probe MAP40160P: FAM- TGTGATGATAAGAACGAAAATTCACAAA-BHQ1 IAC = MAIACP: VIC-CCATACACATAGGTCAGG-MGB-NFQ	300 nM 200 nM	50 cycles of 95°C/15 sec, 60°C/60 sec
Method 3: Becker <i>et al.</i> , 2012; GenBank Accession no. polC and fus A various: see reference: amplicon sizes: 80/83; 95 bp			
<u>Ma/M. mycoides cluster polC /fusA</u>	F1: GCG-CAT-GCT-ACC-GCT-TAT-GT F2: AAG-GCA-CAT-GCT-ACA-GCT-TAT-GTT-AC Probe: VIC-ATGGCACTATGAATAGC-NFQ FusA F: CAA-AAA-GAR-CGT-GTT-GGW-CGT-A FusA R: CCA-ACA-GCT-GCA-GCA-ATA-TC MGB Probe: FAM-CGT-ACT-GAA-ATT-GAA-GAA-G-NFQ	200 and 400 nM 150 to 200 nM 200 and 400 nM 150 to 200 nM	45 cycles of: 95°C/15 sec and 60°C/60 sec

^(a)A denaturation step prior to cycling has not been included.

BHQ1, NFQ – non fluorescent quencher to eliminate background fluorescence.

MGB – minor groove binder probe stabilises binding and improve sequence specificity

1.5.1. Polymerase chain reaction assays

PCR assays are routinely used in many laboratories and are extremely sensitive. They can provide a rapid early warning system when carried out on clinical samples, enabling a full investigation to take place when results are positive. However negative results should not be considered definitive, as culture of bulk tank milk samples may be more sensitive (Tardy *et al.*, 2019; Tatay-Dualde *et al.*, 2015). Several PCRs specific for *Ma* have been developed and show similar levels of sensitivity, although they are based on different gene sequences (Table 2) (Dedieu *et al.*, 1995; Marendia *et al.*, 2005; Subrahmaniam *et al.*, 1998; Tola *et al.*, 1997a). They can be used directly on nasal, conjunctival, synovial and tissue samples; they have been used on milk samples where they have been reported to be more sensitive than culture (Tola *et al.*, 1997a), although occasionally undefined inhibitors may interfere with the test. PCRs can also be used, more reliably, on mycoplasmas growing in culture; a 24 hour enrichment of the mycoplasma in the appropriate medium greatly facilitates PCR detection even in the presence of bacterial contamination (Nicholas, 2002). The use of immunomagnetic capture PCR methods may be more rapid than culture enrichment for detecting *Ma* from milk samples (Sanna *et al.*, 2014). The PCR based on the *uvrC* gene has been evaluated in several laboratories (Bashiruddin *et al.*, 2005). Other molecular methods referred to in this chapter for *Ma* and other mycoplasmas previously added to the aetiology of CA are included in Table 2 as they have been reported widely. The PCRs should be optimised in each laboratory. Positive and negative control DNA should be run in each assay.

A PCR-based method that uses denaturing gradient gel electrophoresis (DGGE) and mycoplasma-specific primers is capable of identifying the majority of small ruminant mycoplasmas, differentiating *Ma* from *Mmc*, *Mcc* and *Mp* including all the causative agents of contagious agalactia by their migration pattern (McAuliffe *et al.*, 2005). Isothermal PCR methods may be applicable; and has been described for detecting *Ma* (Rakha *et al.*, 2015).

A positive PCR result, particularly in an area previously free of contagious agalactia CA, should be confirmed by isolation and identification of the mycoplasma using standard procedures.

Individual PCRs have been reported for *Mmc* (Bashiruddin, 1998) and *Mcc* (Monnerat *et al.*, 1999) and *Mp* (Nicholas *et al.*, 2008; Peyraud *et al.*, 2003) (Table 2), respectively. In addition, A multiplex test has been described that can detect simultaneously *Ma*, *Mcc* and *Mmc* (Greco *et al.*, 2001), while Cillara *et al.* (2015) described a PCR and restriction fragment length polymorphism PCR method based on the *lpdA* gene to discriminate between *Mmc* and *Mcc*. Alternatively, PCR amplification and sequencing of the *fusA* gene can enable differentiation of these latter two organisms (Manso-Silvan *et al.*, 2007).

1.5.2. Real-time PCRs

Several Rapid real-time PCRs have been reported for *Ma* that provide advantages of speed, sensitivity and sample handling (Table 3) (Lorusso *et al.*, 2007). More recently A multiplex real time test has been described which detects all four mycoplasmas simultaneously (Becker *et al.*, 2012), differentiating *Ma* from the mycoplasmas found in goats belonging to the *M. mycoides* cluster and is available in a commercial format.

1.5.3. Micro-array analysis Isothermal molecular assays

Isothermal PCR methods including loop-mediated isothermal amplification (LAMP) may be applicable; and have been described for detecting *Ma* and recently been commercialised, with a portable instrument and kit developed for specific detection of *Ma*. Direct testing of milk samples in the field has shown early promise (Tumino *et al.*, 2020).

Micro-array analysis has been applied to the detection of mycoplasmas. Using probes derived from the 23 rRNA genes and *tuf* gene target regions, Schnee *et al.* (2012) have described the identification of 37 mycoplasma species including all four contagious agalactia pathogens. At the time of publication some cross reaction was seen between *Ma* and the closely related bovine pathogen, *M. bovis*. The advantages of the test over PCR include ease of operation, high information content and cost effectiveness.

1.5.4. Matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF)

Matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF) has been used to identify bacteria (Randall *et al.*, 2015), including *Mycoplasma* species isolated from animals and the causative organisms of contagious agalactia CA (Péreyre *et al.*, 2013; Spengler *et al.*, 2019). The organisms need to be cultured first, and sometimes only the predominant species is identified in mixed cultures. There is not yet an internationally accessible comprehensive database for veterinary mycoplasmas.

1.5.5. Test procedure

The PCR detailed for *Myoplasma agalactiae* has been evaluated in several laboratories (Bashiruddin *et al.*, 2005), whereas other molecular methods referred to in this chapter for *M. agalactiae* and other contagious agalactia causing species have not been evaluated to the same extent, and are therefore not detailed. The PCRs may need to should be optimised in each laboratory. Positive and negative control DNA should be run in each assay.

MAGAUVR1 L ——— CTC AAA AAT ACA TCA ACA AGC

MAGAUVR1 R ——— CTT CAA CTG ATG CAT CAT AA

i) — Extract DNA from *Mycoplasma* isolates or clinical material using the appropriate method (Bolske *et al.*, 1988).

ii) — Carry out PCR methods in 50 µl reaction mixtures containing: 1 µl of sample DNA, 20 pmol of each primer (see above), 1 mM each dNTP, 10 mM Tris/HCl, pH 8.3, 1.5 mM MgCl₂, 50 mM KCl and 1.25 mM U Taq DNA polymerase.

- ~~ii) Subject the mixture to 35 amplification cycles in a thermal cycler with the following parameters: 30 seconds at 94°C, 30 seconds at 50°C annealing temperature and 1 minute at 72°C.~~
- ~~iv iii) Analyse the PCR products by electrophoresis on a 0.7% agarose at 110 V for 2 hours and visualise by staining with ethidium bromide. A 1.7 kb fragment is indicative of the presence of *Ma*.~~

2. Serological tests

2.1. Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assays (ELISAs) using sonicated or Tween-20-treated antigens have been reported to be more sensitive than the CFT for the detection of antibody to *Ma* in serum (Bergonier *et al.*, 1997). Problems of non-specificity have been overcome by the use of monoclonal or protein G conjugates in the ELISA (Lambert *et al.*, 1998). The use of these conjugates enables the testing of sera from a wide range of mammalian species, including camelids. However, whole or refined cell antigen ELISAs are not currently available commercially.

Only one ~~Two~~ commercial ELISA kits for *Ma* are is currently widely available, which uses one using a fusion protein (sensitivity 54 84.6% and specificity 100%) and the other using whole cells as target antigens (sensitivity 84% and specificity 96% in sheep or 90% in goats) (Poumarat *et al.*, 2012 Sánchez *et al.*, 2022). ~~There also appeared to be differences in the ability of the tests to detect the responses to different strains equally. The choice of test depends on the objectives of the proposed study, i.e. a less sensitive test would be sufficient for prevalence study where the disease was endemic while a more sensitive test would be required for disease detection in a disease-free region.~~

ELISAs are not widely available for detecting exposure to *Mmc*, *Mcc* and *Mp* ~~the other three causative mycoplasmas.~~

2.2. Complement fixation

A standard complement fixation test (CFT) for *Ma* has also been applied to *Mmc* and *Mcc* ~~other mycoplasmas involved in the contagious agalactia syndrome (Bergonier *et al.*, 1997), but is now rarely used for *Ma*.~~ Antigens are prepared from washed organisms, standardised by opacity, and lysed, either ultrasonically or by using sodium lauryl sulphate followed by dialysis. Sera are inactivated at 60°C for 1 hour, and the test is carried out in microtitre plates with overnight fixation in the cold or at 37°C for 3 hours. The haemolytic system is added, and the test is read after complete lysis is shown by the antigen control. A positive result is complete fixation at a serum dilution of 1/40 or greater for the following mycoplasmas: *Ma*, *Mcc*, and *Mmc*. The CFT is regarded as a herd test and at least ten sera are tested from each herd, preferably from acute and convalescent cases.

Some sera from healthy flocks react in the CFT using *Ma* up to a serum dilution of 1/20, but rarely react with the other two antigens. However, in flocks infected with *Ma*, sera giving a homologous reaction at 1/80 may cross-react at up to 1/40, the positive threshold, with the other two antigens. It is often difficult to perform the CFT if the quality of the test sera is poor; ~~where possible,~~ the ELISA is preferred for *Ma*.

2.3. Immunoblotting test

Immunoblotting tests have also been described for *Ma* and are considered as confirmatory tests for outbreaks in Italy (Nicholas, 1998; Tola *et al.*, 1997b). Strong bands at approximately 80 and 55 kDa were seen with sera with antibodies to *Ma*, while sera from healthy flocks show no bands or very faint bands of different sizes. Diluting the sera to 1/50 improves the discrimination between positive and negative sera (Nicholas, 1998). On the other hand, Poumarat *et al.* (2012), using a 1/5 dilution of serum, considered a serum positive for *Ma* from a flock/herd in France if it contained 4 bands: 80, 48, 40 and 30 kDa suggesting there may be some geographic differences in humoral responses.

C. REQUIREMENTS FOR VACCINES

Vaccines for the prevention of contagious agalactia-CA due to *Ma* are used widely in the Mediterranean countries of Europe and in western Asia. No single vaccine has been universally adopted, and no standard methods of preparation and evaluation have been applied.

1. Vaccines for *Mycoplasma agalactiae* infection

1.1. Inactivated vaccines for *Mycoplasma agalactiae* infection

In Europe where live vaccines for *Ma* are not acceptable, attention has focused on the use of killed organisms, mostly using formalin and an adjuvant such as aluminium hydroxide in an oil emulsion. The titres of the preparations, before inactivation, are very high (10^8 – 10^{10} colony-forming units per ml) and are derived from laboratory strains. Some products are available commercially, including a trivalent preparation containing *Ma*, *Mcc* and *Mmc* but ~~there are few data~~ are still lacking on their efficacy (Dudek *et al.*, 2022). Autogenous vaccines prescribed for farms with confirmed outbreaks are also used. A formalin-inactivated oil emulsion vaccine was shown to be immunogenic and protective in a small trial in lactating sheep and also prevented transmission of *Ma* (Greco *et al.*, 2002). However, in a small trial, no potency was evident in a similar commercial product following contact challenge (Agnone *et al.*, 2013). Further limitations of current vaccines include a requirement to re-vaccinate, typically at 6-monthly intervals, and failure to prevent intermittent shedding of the pathogen in milk during lactation.

It is possible that in some instances the apparent lack of protection given by vaccines could be the result of animals being infected with *Mmc* or *Mcc* ~~one of the other four mycoplasmas involved in the contagious agalactia syndrome~~ (Gil *et al.*, 1999). A multivalent formalin inactivated vaccine incorporating all four causative mycoplasmas and adjuvanted with saponin and aluminium hydroxide ~~appears to be beneficial in preliminary trials~~ (Ramirez *et al.*, 2001).

~~More recently~~ Vaccines inactivated with phenol or with saponin have given superior protection against experimental infections compared with formalin, sodium hypochlorite or heat-inactivated vaccines (Tola *et al.*, 1999).

1.2. Live attenuated vaccines for *Mycoplasma agalactiae* infection

Live attenuated vaccines against *Ma* have been used in Turkey for many years and have been reported to provide better protection in ewes and their lambs than inactivated vaccines (Nicholas, 2002; Ozdemir *et al.*, 2019). However, they can produce a transient infection with shedding of mycoplasma. Live vaccines should not be used in lactating animals and should be part of a regional plan in which all flocks from which animals are likely to come into contact be vaccinated at the same time.

2. Vaccines for *Mycoplasma mycoides* subsp. *capri* infection

~~There is little recent published information on the availability of vaccines for *Mmc* (Marogna *et al.*, 2015) although it is believed that inactivated vaccines are widely used in many Mediterranean countries and in Asia suggesting that their production and use is localised (Bergonier *et al.*, 1997). Saponised vaccines have been reported in India which provoke a strong antibody response and show some protection (Sunder *et al.*, 2002).~~

3. *Mycoplasma capricolum* subsp. *capricolum* and *M. putrefaciens*

~~Although infections with *Mcc* and *Mp* can be severe, their prevalence is relatively low and, as might be expected, little or no work appears to have been carried out on preventive vaccination for these infections.~~

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NB: There are WOA Reference Laboratories for contagious agalactia (please consult the WOA Web site: <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3> <http://www.oie.int/>).

Please contact the WOA Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for contagious agalactia

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2018.

Appendix 1: Contagious agalactia
Intended purpose of test: population freedom from infection

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
Culture and identification of organism ☒ ☒ ☐	Milk, (individual, pooled, bulk tank) ear swabs, nasal swabs; isolation of <i>Ma</i> (and differentiation from <i>Mmc</i> , <i>Mcc</i> and <i>Mp</i>)	Historical gold standard test		Historical information with no formal validation. See references	1. Demonstrates shedding of viable pathogen 2. Milk collection non-invasive	1. Shedding of pathogen is intermittent in absence of clinical signs 2. Most effective undertaken regularly 3. Bulk tank milk may be insufficiently sensitive as sample type for this purpose 4. Expensive, with significant time required to negate presence of pathogen	Bergonier <i>et al.</i> (1997) Deeney <i>et al.</i> (2021)
Conventional PCR ☒ ☒ ☐	Milk (individual, pooled and bulk milk), ear swabs, nasal swabs; detection of <i>Ma</i> and differentiation from <i>Mmc</i> , <i>Mcc</i> and <i>Mp</i> (various gene targets)	For a range of <i>M. agalactiae</i> tests accuracy ranged from 87 to 100% depending on assay	Largely through method comparisons and inter-laboratory comparisons. Test outlined in the chapter text has been evaluated across several laboratories. PCR-DGGE: continuous passive surveillance of small ruminant mastitis CA in Great Britain	See references	1. Wide applicability 2. Simple yes/no answer based on visual detection of PCR product 3. Milk collection non-invasive	1. Best used in combination with primary or full culture to minimise inhibition 2. Lack of sensitivity for low load samples (typically 10 ² –10 ³ CFU lower sensitivity) compared with real-time PCR 3. Some methods require multi-step confirmatory approach, particularly where organisms belonging to <i>M. mycoides</i> cluster are being considered	Bashiruddin <i>et al.</i> (2005) Tola <i>et al.</i> (1997a) Deeney <i>et al.</i> (2021)
Real-time PCR ☒ ☒ ☐	Milk (individual, pooled, bulk tank), ear swab, nasal swab; detection of <i>Ma</i> (P40, <i>poIC</i> , P80 or other gene targets); <i>fusA</i> detects <i>M. mycoides</i> cluster thus differentiating from <i>Ma</i>	Depending in the assay ASe 10 fg of genomic DNA or <10 genome copies/10 ⁴ CFU/ml in milk – DSe 250 cells in 25 ml of raw milk; up to 100% analytical sensitivity	Performance of the majority of PCRs in use demonstrated under field conditions mostly in areas where CA is known to be present either sporadically or endemically, but including positive and negative flocks/herds: 1. 373 natural raw sheep milk tank samples 2. 465 bulk milk samples over 2 years from 20 goat herds 3. 49,270 samples from 185 herds over 4 yr in endemic area 4. From a disease-free area – 100 lactating does from three herds	See references	1. Kits available that differentiate between <i>Ma</i> and <i>M. mycoides</i> cluster organisms 2. Rapid 3. Can be used directly from samples if validated 4. High sensitivity compared with conventional PCR 5. Suitable for high throughput 6. Milk collection non-invasive	1. Specialist equipment 2. Sensitivity may depend on DNA extraction protocol 3. PCR should be validated for the samples used to determine suitable for testing for this purpose in normally free population, particularly for sensitivity in bulk milk 4. Commercial kits are relatively expensive	Oravcová <i>et al.</i> (2009) Tardy <i>et al.</i> (2019) de la Fe <i>et al.</i> (2025) Fitzmaurice <i>et al.</i> (2008)

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
CFT ☒ ☒ ☒ *	Serum; <i>Ma</i> whole cell antigen (separate tests for <i>Mmc</i> and <i>Mcc</i>)	Historical reference test	N/A	Historical information with no formal validation	1. Detects the earlier presenting IgM as well as IgG, so may detect exposure earlier than IgG based ELISA	1. Considered herd level only 2. Cross reactivity – any non-negative should be followed up by additional testing 3. Test threshold applied could miss healthy carriers	Bergonier <i>et al.</i> (1997)
ELISA ☒ ☒ ☒ **	Serum, plasma; <i>Ma</i> rP48 (or whole cell antigen)	For P48 recombinant antigen-based tests: DSp 100%; DSe 76.9–84.6%. For cellular antigen-based test (no longer commercially available): DSp 100%; DSe 79%. a previous study show DSp ranging from 93.9 to 99.9, with the higher figures in the range associated with sheep	1. 367 sera samples from four herds without a history of CA in the two years prior to sampling 2. 5900 animals from 211 farms with continuous CA monitoring for 20 years 3. 951 serum samples of sheep and goats in disease free country	See references For in-house whole cell antigen-based tests historical validation information only may be available	1. Recombinant antigen ELISA has high specificity 2. Rapid and easy to perform 3. Suitable for high throughput	1. Measures only IgG response which takes some time to develop after onset of infection 2. Herd level test; multiple animals should be tested in each herd 3. Commercial kits are relatively expensive 4. Some whole cell antigen based ELISAs may be less specific	Sánchez <i>et al.</i> (2022) Poumarat <i>et al.</i> (2012) Dudek <i>et al.</i> (2012)
Immunoblotting ☒ ☐ ☐	Serum; <i>Ma</i> whole cell antigen (separate tests with <i>Mcc</i> , <i>Mmc</i> antigens)	Considered historical test	Assessed mainly by comparison with other serological tests, which includes use of field samples.	Historical information with no formal validation See references	1. Most useful as confirmatory screening test	1. Lengthy procedure 2. Impractical for mass screening 3. Interpretation can be subjective – requires specialist knowledge 4. Difficult to standardise	Nicholas (1998) Poumarat <i>et al.</i> (2012) de la Fe <i>et al.</i> (2006)

*Only recommended for *Mcc* and *Mmc*; **Only available for *Ma*; ~Some ELISA kits state suitable for use with plasma, but this should be validated in-house.

Appendix 2. Contagious agalactia
Intended purpose of test: individual animal freedom from infection

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Culture and identification of organism</u> ☒ ☒ ☒	<u>Individual milk, ear swabs, nasal swabs (semen^a, preputia^a, vaginal swab^a) isolation of <i>Ma</i> (and differentiation from <i>Mmc</i>, <i>Mcc</i> and <i>Mp</i>)</u>	<u>Historical gold standard test</u>		<u>Historical information with no formal validation</u> <u>See references</u>	<u>1. Demonstrates shedding of viable pathogen</u> <u>2. Milk collection non-invasive</u>	<u>1. Shedding of pathogen may be intermittent in absence of clinical signs in regions where disease is endemic</u> <u>2. Most effective undertaken regularly</u> <u>3. Time required to negate pathogen</u>	<u>Bergonier <i>et al.</i> (1997)</u>
<u>Conventional PCR</u> ☒ ☒ ☒	<u>Individual milk, ear swabs, nasal swabs: detection/identification of <i>Ma</i> (various gene targets) and differentiation from <i>Mmc</i>, <i>Mcc</i> and <i>Mp</i></u>	<u>For a range of <i>Ma</i> tests accuracy ranged from 87–100% depending on assay</u>	<u>Method comparisons inter-laboratory comparisons</u> <u>Test outlined in the chapter text has been evaluated across several laboratories</u>	<u>See references</u>	<u>1. Wide applicability</u> <u>2. Simple yes/no answer based on visual detection of PCR product</u> <u>3. Milk collection non-invasive</u>	<u>1. Best used in combination with primary or full culture to minimise inhibition</u> <u>2. Lack of sensitivity for low load samples (typically 10²–10³ CFU lower sensitivity) compared with PCR depending on method.</u> <u>3. Some methods require multi-step confirmatory approach, particularly where organisms belonging to <i>M. mycoides</i> cluster need to be considered</u> <u>4. For endemic areas multiple samples over time required</u>	<u>Bashiruddin <i>et al.</i> (2005)</u> <u>Tola <i>et al.</i> (1997a)</u> <u>Dedieu <i>et al.</i> (1995)</u> <u>Peyraud <i>et al.</i> (2003)</u> <u>Cillara <i>et al.</i> (2015)</u>
<u>Real-time PCR</u> ☒ ☒ ☒	<u>Individual milk (ear swab, nasal swab, eye swab): detection/identification of <i>M. agalactiae</i>. detection of <i>M. agalactiae</i> (P40, <i>polC</i>, P80 or other gene targets); <i>fusA</i> detects <i>M. mycoides</i> cluster thus differentiating from <i>Ma</i></u>	<u>Depending on analytical sensitivity of the assay: ASe 10 fg of genomic DNA or <10 genome copies/10⁴ CFU/ml in milk – DSe 250 cells in 25 ml of raw milk; up to 100% ASe</u>	<u>Performance of the majority of PCRs developed has been demonstrated under field conditions mostly in areas where CA is known to be present either sporadically or endemically, but including positive and negative flocks/herds:</u> <u>1. 373 natural raw sheep milk tank samples</u> <u>2. 49,270 samples from 185 herds over 4 years in endemic area.</u> <u>3. From a disease-free area – 100 lactating does from three herds</u>	<u>See references</u>	<u>1. Kits available that differentiate between <i>Ma</i> and <i>M. mycoides</i> cluster organisms</u> <u>2. Rapid</u> <u>3. Can be used directly from samples</u> <u>4. High sensitivity compared with conventional PCR</u> <u>5. Amenable to high throughput</u> <u>6. Milk collection non-invasive</u>	<u>1. Specialised equipment</u> <u>2. Sensitivity may depend on DNA extraction protocol</u> <u>3. PCR should be validated for the samples used to determine suitable for testing for respective sample types</u> <u>4. Commercial kits are relatively expensive</u>	<u>Oravcová <i>et al.</i> (2009)</u> <u>de la Fe <i>et al.</i> (2025) (original method</u> <u>Becker <i>et al.</i> [2012])</u> <u>Fitzmaurice <i>et al.</i> (2008)</u>
<u>CFT</u> ☒ ☐ ☐ ☐ *	<u>Serum; antibodies indicating exposure to <i>M. agalactiae</i></u>	<u>Historical reference test</u>	<u>N/A</u>	<u>Historical information with no formal validation</u> <u>See reference</u>	<u>1. Detects the earlier presenting IgM as well as IgG, so may detect exposure earlier than IgG based ELISA</u>	<u>1. Considered herd level only; minimal ten animals</u>	<u>Bergonier <i>et al.</i> (1997)</u>

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
	(separate tests for <i>Mmc</i> and <i>Mcc</i>)					<u>2. Cross reactivity – any non-negative should be followed up by additional testing</u> <u>3. Test threshold applied could miss healthy carriers</u>	
<u>ELISA</u> ☒ ☐ ☐ **	Serum, plasma; rP48, (or whole cell antigen)	<u>For P48 recombinant antigen-based tests: DSp 100%; DSe 76.9–84.6%.</u> <u>For cellular antigen-based test (no longer commercially available): DSp 100%; DSe 79%, a previous study show DSp ranging from 93.9 to 99.9, with the higher figures in the range associated with sheep</u>	<u>367 sera samples from four herds without a history of CA in the 2 years prior to sampling</u> <u>5900 animals from 211 farms with continuous CA monitoring for 20 years</u> <u>951 serum samples of sheep and goats in disease free country</u>		<u>1. Recombinant antigen ELISA has high specificity</u> <u>2. Rapid and easy to perform</u>	<u>1. Herd level test; multiple animals should be tested in each herd</u> <u>2. Measures only IgG response which takes some time to develop after onset of infection</u> <u>3. Commercial kits are relatively expensive</u> <u>4. Some whole cell antigen based ELISAs may be less specific</u>	<u>Sánchez <i>et al.</i> (2022)</u> <u>Poumarat <i>et al.</i> (2012)</u> <u>Dudek <i>et al.</i> (2012)</u>
<u>Immunoblotting</u> ☒ ☒ ☐	Serum; <i>Ma</i> whole cell antigen (also used with <i>Mcc</i> , <i>Mmc</i> whole cell antigens)	<u>Considered historical test</u>	<u>Mainly assessed by comparison with other serological tests but includes use of field samples</u>	<u>Historical information with no formal validation</u> <u>See references</u>	<u>1. Suitable for individual animals</u> <u>2. Most useful as confirmatory screening test</u> <u>3. Suited to high throughput</u>	<u>1. Lengthy procedure</u> <u>2. Impractical for mass screening</u> <u>3. Interpretation can be subjective – requires specialist knowledge</u> <u>4. Difficult to standardise</u>	<u>Nicholas (1998)</u> <u>Poumarat <i>et al.</i> (2012)</u> <u>de la Fe <i>et al.</i> (2006)</u>

*Only recommended for *Mcc* and *Mmc*; **Only available for *Ma*; ~Some ELISA kits state suitable for use with plasma, but this should be validated in-house; #Animals intended for breeding/trade of germplasm.

Appendix 3: Contagious agalactia
Intended purpose of test: contribute to eradication policies

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
Culture and identification of organism ☒ ☒ ☒	Individual and bulk milk, ear swabs, nasal swabs (semen ^a , preputia ^a , vaginal swab ^a) isolation of <i>Ma</i> (and differentiation from <i>Mmc</i> , <i>Mcc</i> and <i>Mp</i>)	Historical gold standard test		Historical information with no formal validation	1. Demonstrates shedding of viable pathogen and current infection 2. Milk collection non-invasive	1. Should be used in combination with serology for this purpose 2. Shedding of pathogen is intermittent in absence of clinical signs 3. Most effective undertaken regularly 4. Time and cost to monitor sufficient animals	Bergonier <i>et al.</i> (1997)
Real-time PCR ☒ ☐ ☐	Individual milk, ear swab, nasal swab: detection/identification of <i>Ma</i> (various gene targets P40, polC, P80 or other gene targets); <i>fusA</i> detects <i>M. mycoides</i> cluster thus differentiating from <i>Ma</i>	Depending on analytical sensitivity of the assay: ASe 10 fg of genomic DNA or <10 genome copies/10 ⁴ CFU/ml in milk – DSe 250 cells in 25 ml of raw milk; up to 100% ASe	Performance of the majority of PCRs developed has been demonstrated under field conditions mostly in areas where CA is known to be present either sporadically or endemically but including positive and negative flocks/herds	See references	1. Kits available that differentiate between <i>Ma</i> and <i>M. mycoides</i> cluster organisms 2. Rapid 3. Higher sensitivity compared with conventional PCR 4. Suited to high throughput 5. Milk collection non-invasive	1. Specialist equipment 2. In combination with serology for this purpose 3. Commercial kits are relatively expensive	de la Fe <i>et al.</i> (2025) Tardy <i>et al.</i> (2019). https://www.anses.fr/fr/system/files/LABO-Ra-Vigimyc2022.pdf
CFT ☒ ☒ ☒ *	Serum: antibodies indicating exposure to <i>Ma</i> (alternative antigens for <i>Mmc</i> and <i>Mcc</i>)	Historical reference test	N/A	Historical information with no formal validation	1. detects the earlier presenting IgM as well as IgG, so may detect exposure earlier than IgG based ELISA	1. Considered herd level only; minimal ten animals 2. Cross reactivity – any non-negative should be followed up by additional testing 3. Test threshold applied could miss healthy carriers	Bergonier <i>et al.</i> (1997)
ELISA ☒ ☒ ☒ **	Serum, plasma; rP48, (or whole cell antigen)	For P48 recombinant antigen-based tests: DSp 100%; DSe 76.9–84.6%. For cellular antigen-based test (no longer commercially available): DSp 100%; DSe 79%, a previous study show DSp ranging from 93.9 to 99.9.	1. Continual surveillance of flocks in the Pyrenees – Atlantique region of France 2. 367 sera samples from four herds without a history of CA in the 2 years prior to sampling 3. 5900 animals from 211 farms with continuous CA monitoring for 20 years	See references	1. Recombinant antigen ELISA has high specificity 2. Rapid and easy to perform 3. Suitable for high throughput	1. Herd level test; multiple animals should be tested in each herd 2. Best in combination with PCR 3. Commercial kits are relatively expensive 4. Some whole cell antigen based ELISAs may be less specific	Les actions de l'Agalactie Contagieuse Sánchez <i>et al.</i> (2022) Poumarat <i>et al.</i> (2012)

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
		<u>with the higher figures in the range associated with sheep</u>					
<u>Immunoblotting</u> ☒ ☐ ☐	<u>Serum: <i>Ma</i> whole cell antigen (also used with <i>Mcc</i>, <i>Mmc</i> whole cell antigens)</u>	<u>Considered historical test</u>	<u>Mainly assessed by comparison with other serological tests</u>	<u>Historical information with no formal validation</u> <u>See references</u>	<u>1. Most useful as confirmatory screening test</u>	<u>1. Lengthy procedure</u> <u>2. Impractical for mass screening</u> <u>3. Interpretation can be subjective - requires specialist knowledge</u> <u>4. Difficult to standardise</u>	<u>Poumarat <i>et al.</i> (2012)</u> <u>de la Fe <i>et al.</i> (2006)</u>

*Only recommended for *Mcc* and *Mmc*; **Only available for *Ma*; ~Some ELISA kits state suitable for use with plasma, but this should be validated in-house; #Animals intended for breeding.

Appendix 4: Contagious agalactia
Intended purpose of test: confirmation of clinical cases

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Culture and identification of organism</u> ☒ ☒ ☒	<u>Individual milk, ocular swab, joint fluid, nasal swab, other fluids/tissues according to clinical signs and whether live or dead animals (including abortifacient material, blood, udder, lymph nodes, lung); isolation of <i>Ma</i> and distinguishing from <i>Mcc</i>, <i>Mmc</i> and <i>Mp</i> as relevant</u>	<u>Historical gold standard test</u>		<u>Historical information with no formal validation</u>	<u>1. Demonstrates shedding of viable pathogen</u> <u>2. Reduces background from sample matrix that can interfere with PCR</u> <u>3. Ensures mycoplasma-like organism is identified, enabling complementary testing and strain confirmation</u> <u>4. Recommended requirement following a positive PCR test in an area previously free of contagious agalactia</u>	<u>1. Requirement for specialist media to minimise overgrowth of contaminants or other pathogens that may be associated with similar clinical signs</u> <u>2. Further identification methods such as PCR are still required</u> <u>3. Isolation of pure colony for downstream identification can be lengthy process</u>	<u>Bergonier <i>et al.</i> (1997)</u>
<u>Conventional PCR</u> ☒ ☒ ☒	<u>Individual milk, ocular swab, joint fluid, nasal swab, other fluids/tissues according to clinical signs and whether live or dead animals (including abortifacient material, blood, udder, lymph nodes, lung); detection/identification of <i>Ma</i> (various gene targets). Differentiation from <i>Mmc</i>, <i>Mcc</i>, <i>Mp</i> as relevant</u>	<u>For a range of <i>Ma</i> tests accuracy ranged from 87–100% depending on assay</u>	<u>Method comparisons inter-laboratory comparisons using panels of 53–100 isolates (Cillara <i>et al.</i>, 2015; Dedieu <i>et al.</i>, 1995; Peyraud <i>et al.</i>, 2003) Test outlined in the chapter text has been evaluated using panels across several laboratories as has that of Woubit <i>et al.</i> (2007) for <i>M. mycoides</i> cluster, which also included 21 natural milk samples 357 samples from 21 newly infected flocks (group 1) and 87 samples from eight flocks previously experiencing outbreaks (Tola <i>et al.</i>, 1997a) 255 clinical mastitis samples from 80 dairy goats (Tatay-Dualde <i>et al.</i>, 2015)</u>	<u>See references</u>	<u>1. Wide applicability</u> <u>2. Simple yes/no answer based on visual detection of PCR product</u>	<u>1. Best used in combination with primary or full culture to minimise inhibition</u> <u>2. Lack of sensitivity for low load samples (typically 10²–10³ CFU lower sensitivity) compared with real-time PCR depending on method</u> <u>3. Some methods require multi-step confirmatory approach, or sequencing of product, particularly where organisms belonging to <i>M. mycoides</i> cluster are being considered</u>	<u>Bashiruddin <i>et al.</i> (2005)</u> <u>Tola <i>et al.</i> (1997a)</u> <u>Dedieu <i>et al.</i> (1995)</u> <u>Tatay-Dualde <i>et al.</i> (2015)</u> <u>Peyraud <i>et al.</i> (2003)</u> <u>Woubit <i>et al.</i> (2007)</u> <u>Cillara <i>et al.</i> (2015)</u>
<u>Real-time PCR</u> ☒ ☒ ☒	<u>Individual milk, ocular swab, joint fluid, nasal swab, other fluids/tissues</u>	<u>Depending on analytical sensitivity of the assay: ASe 10 fg</u>	<u>Performance of the majority of PCRs developed has been demonstrated under field conditions mostly in areas</u>	<u>See references.</u>	<u>1. Kits available that differentiate between <i>Ma</i> and <i>M. mycoides</i> cluster organisms</u> <u>2. Rapid</u>	<u>1. Specialist equipment</u> <u>2. Sensitivity may depend on DNA extraction protocol</u>	<u>Oravcová <i>et al.</i> (2009)</u> <u>Becker <i>et al.</i> (2012)</u>

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
	according to clinical signs and whether live or dead animals (including abortifacient material, blood, udder, lymph nodes, lung: detection/identification of <i>Ma</i> (P40, polC, P80 or other gene targets); <i>fusA</i> detects <i>M. mycoides</i> cluster thus differentiating from <i>Ma</i>	of genomic DNA or <10 genome copies/10 ⁴ CFU/ml in milk – DSe 250 cells in 25 ml of raw milk; up to 100% ASE	where CA is known to be present either sporadically or endemically, but including positive and negative flocks/herds: –424 milk samples from individual sheep from an infected flock		3. Can be used directly from samples if validated 4. High sensitivity compared with conventional PCR 5. Suitable for high throughput	3. PCR should be validated for the samples used to determine suitability of cut-off value for respective sample types 4. Commercial kits are expensive	
CFT ☒ ☒ ☒ *	Serum; antibodies indicating exposure to <i>Ma</i> (separate tests for <i>Mmc</i> and <i>Mcc</i>)	Historical reference test	N/A	Historical information with no formal validation	1. Detects the earlier presenting IgM as well as IgG	1. In combination with antigen testing as considered herd level test 2. Cross reactivity – any non-negative should be followed up by additional testing 3. Test threshold applied could miss healthy carriers 4. Difficult to perform if quality of test sera is poor 5. Does not differentiate between vaccinated and naturally infected animals	Bergonier <i>et al.</i> (1997)
ELISA ☒ ☒ ☒ **	Serum, plasma; rP48, (or whole cell antigen)	For P48 recombinant antigen-based tests: DSp 100%; DSe 76.9–84.6%. For cellular antigen-based test (no longer commercially available): DSp 100%; DSe 79%, a previous study show DSp ranging from 93.9 to 99.9, with the higher	1. 394 sera samples from herds with a previous history of clinical cases of CA including acute and chronic clinical cases 2. 5900 animals from 211 farms with continuous CA monitoring for 20 years		1. Recombinant antigen ELISA has high specificity 2. Rapid and easy to perform 3. Suitable for high throughput	1. Herd level test – multiple animals. Best in combination with antigen testing 2. Measures only IgG response which takes some time to develop after onset of infection 3. Commercial kits are relatively expensive 4. Some in house whole cell antigen based ELISAs may be less specific 5. Does not differentiate between vaccinated and naturally infected animals	Sánchez <i>et al.</i> (2022) Poumarat <i>et al.</i> (2012)

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
		<u>figures in the range associated with sheep</u>					
Immunoblotting ☒ ☒ ☐	Serum: <u>Ma whole cell antigen</u> (separate tests for <u>Mcc</u> , <u>Mmc</u> whole cell antigens)	<u>Considered historical test</u>	Mainly assessed by <u>comparison with other serological tests</u> but includes <u>field samples</u>	Historical information with no formal validation -See references	1. Suitable for individual animals 2. Particularly useful as confirmatory screening test	1. Lengthy procedure 2. Interpretation can be subjective - requires specialist knowledge 3. Doesn't differentiate between vaccinated and naturally infected animals 4. Difficult to standardise	<u>Poumarat <i>et al.</i> (2012)</u> <u>de la Fe <i>et al.</i> (2006)</u>

*Only recommended for Mcc and Mmc; **Only available for Ma; ~Some ELISA kits state suitable for use with plasma, but this should be validated in-house.

Appendix 5: Contagious agalactia
Intended purpose of test: prevalence of infection – surveillance

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
Culture and identification of organism ☒ ☐ ☐	individual and bulk milk, ocular swab, joint fluid, nasal swab: isolation of <i>Ma</i> (differentiation from <i>Mmc</i> , <i>Mcc</i> , <i>Mp</i>)	Historical gold standard test	1. Monthly bulk tank milk samples from 20 farms over 2 years (Tardy <i>et al.</i> , 2019) 2. Sheep milk samples from 922 farms (Ariza-Miguel <i>et al.</i> , 2012) 3. Ongoing surveillance	See references	1. Demonstrates shedding of viable pathogen 2. Reduces background from sample matrix that can interfere with PCR 3. Ensures mycoplasma-like organism is identified, enabling complementary testing and strain confirmation 4. Bulk milk testing is non-invasive way of screening herd	1. Isolation of pure colony for downstream identification, such as by PCR can be lengthy process, thus expensive for surveillance except on small scale	Bergonier <i>et al.</i> (1997) Tardy <i>et al.</i> (2019) Ariza-Miguel <i>et al.</i> (2012) Vigimyc network Anses - Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail
Conventional PCR ☒ ☒ ☐	Individual and bulk milk, ear swabs, (nasal swabs, semen): detection/identification of <i>Ma</i> (various gene targets). Enables differentiation from <i>Mmc</i> , <i>Mcc</i> and <i>Mp</i> as necessary	For a range of <i>M. agalactiae</i> tests accuracy ranged from 87 to 100% depending on assay	Method comparisons inter-laboratory comparisons using panels of 53–100 isolates. Field studies conducted mainly in endemic areas – 681 milk samples from 66 herds representing 12.8% of dairy goats. 1. 357 samples from 21 newly infected flocks (group 1) and 87 samples from eight flocks previously experiencing outbreaks 2. 255 clinical mastitis samples from 80 dairy goats)	See references	1. Wide applicability 2. Simple yes/no answer based on visual detection of PCR product 3. Bulk milk testing is non-invasive way of screening herd	1. Best used in combination with primary or full culture to minimise inhibition 2. Lack of sensitivity for low load samples (typically 10 ² –10 ³ CFU lower sensitivity) compared with real-time PCR depending on method 3. Some methods require multi-step confirmatory approach, or sequencing of product, particularly where organisms belonging to <i>M. mycoides</i> cluster are being considered	Bashiruddin <i>et al.</i> (2005) Dedieu <i>et al.</i> (1995) Peyraud <i>et al.</i> (2003) Woubit <i>et al.</i> (2007) Cillara <i>et al.</i> (2015) Tola <i>et al.</i> (1997a) Tatay-Dualde <i>et al.</i> (2015)
Real-time PCR ☒ ☒ ☐	Bulk/pooled /individual milk, ear swab, (nasal swab, semen): detection/identification of <i>Ma</i> . (P40, polC, P80 or other gene targets); <i>fusA</i> detects <i>M. mycoides</i> cluster thus differentiating from <i>Ma</i>	Depending on analytical sensitivity of the assay: ASe 10 fg of genomic DNA or <10 genome copies/10 ⁴ CFU/ml in milk – DSe 250 cells in 25 ml of raw milk; up to 100% ASe	Performance of the majority of PCRs developed has been demonstrated under field conditions mostly in areas where CA is known to be present either sporadically or endemically, but including positive and negative flocks/herds: 1. 373 natural raw sheep milk tank and 424 individual milk samples 2. Monthly bulk tank milk samples from 20 farms over 2 years	See references	1. Kits available that differentiate between <i>M. agalactiae</i> and <i>M. mycoides</i> cluster organisms 2. Rapid 3. Can be used directly from samples 4. High sensitivity compared with conventional PCR 5. Suitable for high throughput 6. Bulk milk testing is non-invasive way of screening herd	1. Specialist equipment 2. Sensitivity may depend on DNA extraction protocol 3. PCR should be validated for the samples used to determine suitability of cut-off value for respective sample types 4. Commercial kits are expensive	Oravcová <i>et al.</i> (2009) Tardy <i>et al.</i> (2019) Ariza-Miguel <i>et al.</i> (2012) de la Fe <i>et al.</i> (2025)

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
			<u>3. Sheep milk samples from 922 farms</u> <u>4. 681 milk samples from 66 herds representing 12.8% of dairy goats</u>				
<u>LAMP</u> ☒ ☒ ☐	<u>Individual milk; detection of <i>Ma</i> P40 gene</u>	<u>Se 90%; Sp100%</u>	<u>Performance for LAMP has been demonstrated under field conditions for 110 individual milk samples of known positive /negative status in endemic area</u>	<u>See reference</u>	<u>1. Commercially available kit for direct detection of <i>Ma</i> in milk</u> <u>2. Can be performed in real-time in the field</u>	<u>1. Specialist equipment</u> <u>2. Limited numbers of samples at any one time depending on platform</u> <u>3. Method needs wider study in the field</u> <u>4. Difficult to set up in-house assays for field use due to challenges associated with sample preparation outside the lab environment</u>	<u>Tumino <i>et al.</i> (2020)</u>
<u>CFT</u> ☒ ☒ ☒ *	<u>Serum; antibodies indicating exposure to <i>Ma</i> (separate tests for <i>Mmc</i> and <i>Mcc</i>)</u>	<u>Historical reference test</u>	<u>N/A</u>	<u>Historical information with no formal validation</u>	<u>1. Detects the earlier presenting IgM as well as IgG</u>	<u>1. Considered herd level only; multiple animals should be tested</u> <u>2. Cross reactivity – any non-negative should be followed up by additional testing</u> <u>3. Test threshold applied could miss healthy carriers</u> <u>4. Largely discontinued for <i>M. agalactiae</i></u> <u>5. Difficult to perform if quality of test sera is poor</u> <u>4. Does not differentiate between vaccinated and naturally infected animals</u>	<u>Bergonier <i>et al.</i> (1997)</u>
<u>ELISA</u> ☒ ☒ ☒ **	<u>Serum, plasma; rP48, (or whole cell antigen)</u>	<u>For P48 recombinant antigen-based tests: DSp 100%; DSe 76.9–84.6%.</u> <u>For cellular antigen-based test (no longer commercially available): DSp 100%; DSe 79%, a previous study show DSp ranging from 93.9 to 99.9, with the higher figures in the</u>	<u>1. 394 sera samples from herds with a previous history of clinical cases of CA</u> <u>2. 5900 animals from 211 farms with continuous CA monitoring for 20 years</u> <u>3. 24810 samples from six monthly sampling on unvaccinated animals aged 4 years or older over 2 years</u>		<u>1. Recombinant antigen ELISA has high specificity</u> <u>2. Rapid and easy to perform</u> <u>3. Suitable for high throughput</u>	<u>1. Does not differentiate between vaccinated and unvaccinated animals: only for unvaccinated herds/flocks</u> <u>2. Herd level test; multiple animals should be tested in each herd</u> <u>3. Measures only IgG response which takes some time to develop after onset of infection</u> <u>4. Commercial kits are relatively expensive</u> <u>5. Some whole cell antigen-based ELISAs may be less specific</u>	<u>1. Sánchez <i>et al.</i> (2022)</u> <u>2. Poumarat <i>et al.</i> (2012)</u> <u>de la Fe <i>et al.</i> (2025)</u>

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
		<u>range associated with sheep</u>					
Immunoblotting ☒ ☐ ☐	Serum: <u>Ma</u> whole cell antigen (separate tests for <u>Mcc</u> , <u>Mmc</u> whole cell antigens)	Considered historical test	Mainly assessed by comparison with other serological tests but includes field samples	Historical information with no formal validation See references	1. Most useful as confirmatory screening test	1. Lengthy procedure 2. Impractical for mass screening 3. Interpretation can be subjective - requires specialist knowledge 4. Does not differentiate between vaccinated and naturally infected animals 5. Difficult to standardise	Poumarat <i>et al.</i> (2012) de la Fe <i>et al.</i> (2006)

*Only recommended for *Mcc* and *Mmc*; **Only available for *Ma*; ~Some ELISA kits state suitable for use with plasma, but this should be validated in house; ¢Animals intended for breeding/trade of germplasm.

Appendix 6: Contagious agalactia

Intended purpose of test: immune status in individual animals or populations post-vaccination

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>CFT ☒☒☒*</u>	<u>Serum: antibodies indicating exposure to <i>Ma</i> (separate tests for <i>Mmc</i> and <i>Mcc</i>)</u>	<u>Historical test. Performance not determined for this purpose</u>	<u>N/A</u>	<u>See reference</u>		<u>1. Considered herd level test – need to test multiple animals</u> <u>2. Detected titre declines quickly over time following vaccination</u> <u>3. Difficult to perform if quality of test sera is poor</u>	
<u>ELISA ☒☒☒**~</u>	<u>Serum plasma: rP48, (or whole cell antigen)</u>	<u>Not determined for this purpose</u>	<u>1. Experimental trial data – 28 ewes receiving one of four vaccinations (P48 recombinant ELISA)</u> <u>2. Experimental trial involving two commercial <i>Ma</i> vaccines and 700 sheep</u> <u>3. Experimental trial live vaccine on 30 goats</u> <u>4. 65 animals (sheep and goats) receiving <i>Ma</i> vaccination</u>	<u>See Reference</u>	<u>1. Test is rapid and easy to use</u> <u>2. Suitable for high throughput</u>	<u>1. Live vaccines used in Türkiye do not raise a significant protective immune response</u> <u>2. Considered herd level test</u> <u>3. Detected titre declines quickly over time following vaccination</u> <u>4. Does not differentiate between vaccinated and naturally infected animals</u>	<u>Agnone <i>et al.</i> (2013)</u> <u>Mogoş <i>et al.</i> (2021)</u> <u>Ozdemir <i>et al.</i> (2019)</u> <u>Evstatiev <i>et al.</i> (2020)</u>
<u>Immunoblotting ☒☒☐</u>	<u>Serum (separate tests for <i>Mcc</i>, <i>Mmc</i> whole cell antigens)</u>	<u>Not determined for this purpose</u>	<u>N/A</u>	<u>Historical information with no formal validation</u>	<u>1. Most useful as confirmatory screening test</u>	<u>1. Lengthy procedure</u> <u>2. Impractical for mass screening</u> <u>3. Interpretation can be subjective – requires specialist knowledge</u> <u>4. Does not differentiate between vaccinated and naturally infected animals</u> <u>5. Difficult to standardise</u>	

MEETING OF THE BIOLOGICAL STANDARDS COMMISSION

Paris, 8–12 September 2025

CHAPTER 3.10.2.
CYSTICERCOSIS
(INCLUDING INFECTION WITH *TAENIA SOLIUM*)¹

SUMMARY

Description of the disease: Cysticercosis of farmed and wild animals is caused by the larval stages (metacestodes) of cestodes of the family Taeniidae (tapeworms), the adult stages of which occur in the intestine of humans, dogs, cats or wild Canidae and Mustellidae. Bovine cysticercosis (primarily in muscle) and porcine cysticercosis (primarily in muscle and the central nervous system [CNS]) are caused by the metacestodes (cysticerci) of the human cestodes *Taenia saginata* and *T. solium*, respectively. Cysticerci of *T. solium* also develop in the CNS, musculature and subcutaneous tissue of humans. *Taenia asiatica* is a less widespread cause of cysticercosis in pigs, with the cysts locating in the liver and viscera and the adult tapeworm occurring in humans. Cysticercosis and coenurosis of sheep and goats, and occasionally cattle, with the cysts occurring in the muscles, brain, liver or peritoneal cavity, are caused by *T. ovis*, *T. multiceps* and *T. hydatigena*, with the adult tapeworms occurring in the intestines of dogs and wild canids.

Most adult and larval tapeworm infections cause little or no disease. Exceptions are severe, potentially fatal human neurocysticercosis (NCC) caused by *T. solium*, and occasionally neuro-coenurosis caused by *T. multiceps* in humans. These parasites are also occasional causes of muscle or ocular signs in humans. Rare but severe cysticercoses in humans can be caused by the fox tapeworm *Taenia crassiceps* and the mustelid tapeworms *Taenia martis* and *Versteria* spp. 'Gid' caused by *T. multiceps* in ruminants can require surgery or slaughter of the animal. Acute *T. multiceps* coenurosis and *T. hydatigena* cysticercosis in sheep and goats are rare but may be fatal. Cysticercosis causes economic loss through condemnation of infected meat and offal.

Detection of the agent: Adult *Taenia* tapeworms are dorsoventrally flattened, segmented and large, reaching from 20 to 50 cm (species in dogs) to several metres (species in humans). Anteriorly, the scolex (head) has four muscular suckers and may have a rostellum, often armed with two rows of hooks, the length and number of these being relatively characteristic of a species. A neck follows the scolex, and this is followed by immature and then by mature reproductive segments, and finally gravid segments filled with eggs. Segment structure, although unreliable, can aid in the identification of the species. Adult *Taenia* are recognised at post-mortem or by passage of segments or eggs in faeces. *Taenia* species cannot be differentiated by egg structure, detected via coprological techniques in fecal samples. Metacestodes consist of a fluid-filled bladder with one or more invaginated scoleces. These 'bladderworms' are each contained within a cyst wall at the parasite–host interface. This structure comprises the cysticercus or coenurus. Metacestodes are grossly visible at post-mortem and meat inspection, but light infections are often missed. NCC can be diagnosed primarily by imaging techniques.

¹ Although certain diseases caused by *Taeniidae* are included in some individual species sections of the WOAHL, this chapter covers several species and thus gives a broader description.

Immunological and molecular tests: Adult human *Taenia* infections can be diagnosed by detection of *Taenia* coproantigen in faeces using an antigen-capture enzyme-linked immunosorbent assay (Ag-ELISA). Several species-specific DNA-based techniques have been developed that can be applied on parasite material or on faecal extracts, but some remain to be fully validated.

Serological tests: Commercial antibody-detecting tests (enzyme linked immunoelectrotransfer blot) are available for the diagnosis of *T. solium* cysticercosis in people and in pigs, though for the latter the diagnostic performance is limited due to a high false positive rate. A commercial test (ELISA format) is available for detection of circulating parasite-derived antigen in the serum of pigs or humans with *T. solium* cysticercosis, but However, in regions where infection with *T. hydatigena* is common in pigs (e.g. the Americas and South-East Asia) the use of the Ag-ELISA for diagnosis of *T. solium* in pigs is limited due to a high false positive rate and low positive predictive value.

Requirements for vaccines: Excellent vaccines based on recombinant antigens derived from oncosphere proteins have been developed for immunising animals against cysticercosis caused by *T. ovis* in sheep, *T. saginata* in cattle and *T. solium* in pigs. Currently, no vaccines are available for the adult stages of *Taenia* spp. A *T. solium* vaccine for pigs is commercially available; it is approved in some countries and undergoing regulatory approval in other countries. A combination of vaccination and oxfendazole treatment was highly effective in experimental control of natural transmission to pigs. Immunisation of pigs for *T. solium* cysticercosis requires at least two vaccinations.

A. INTRODUCTION

The metacestodes (or larval cestodes) of *Taenia* spp. tapeworms are the cause of cysticercosis in various farmed and wild animals and in humans. Adult tapeworms are found in the small intestine of carnivore definitive hosts: humans, dogs, cats and wild canids and mustelids. *Taenia saginata* of humans causes bovine cysticercosis, which occurs virtually world-wide, but particularly in Africa, Latin America, Caucasian and South/Central Asia and eastern Mediterranean countries. The infection occurs in many countries in Europe and sporadically in the United States of America (USA), Canada, Australia and New Zealand. *Taenia solium* of humans causes porcine cysticercosis and human (neuro)cysticercosis ([N]CC), the most important cause of acquired epilepsy in endemic areas. It is found principally in Mexico, Central and South America, sub-Saharan Africa, non-Islamic countries of Asia, including India and China (People's Rep. of) in regions with poor sanitation and free-ranging, scavenging pigs. In 2016, central nervous system syndromes, including seizures and depressed spirit listlessness, have been observed in pigs naturally infected with *T. solium* (Trevisan *et al.*, 2016). The cysticerci of the human tapeworm *T. asiatica* in South-East Asia occur in the liver of pigs. Dogs and wild canids are the definitive hosts of *Taenia* spp. that have sheep, goats and other ruminants and pigs as intermediate hosts, which occur throughout most of the world, although *T. multiceps* has disappeared from the USA, Australia and New Zealand. *Taenia ovis* cysticerci occur in the muscles of sheep, *T. multiceps* in the brain (occasionally in the muscles) of sheep, goats, sometimes other ruminants and rarely humans, and *T. hydatigena* is found in the peritoneal cavity and on the liver of ruminants and pigs. *Taenia crassiceps*, a species mainly occurring in foxes, causes, though rarely, ocular and neural cysticercoses in immunocompetent human patients and severe subcutaneous disseminated and proliferative cysticercoses in immunodeficient patients; *T. martis*, a tapeworm of martens in Europe causes rare cysticercosis in immunocompetent humans. A *Versteria* sp. distinct from *V. mustelae* caused severe and lethal cysticercoses in heavily immunosuppressed patients in North America (Deplazes *et al.*, 2019). Diagnosis in animals usually is based on identification of the metacestode at meat inspection or necropsy. Adults in definitive hosts are acquired by the ingestion of viable metacestodes in meat and offal that has not been adequately cooked or frozen to kill the parasite.

Gravid segments are shed by the adult tapeworms. Tapeworm eggs, within proglottids or separate, are released into the environment with the faeces. For certain species (*T. saginata*, canid taeniids), active migration of the proglottids out of the anus occurs. Eggs may be disseminated from faeces by physical means or transport hosts. Flies particularly ingest eggs and transport these eggs, so eggs are may be deposited at high intensity within 150 m of the faeces and at low intensity for 10 km (Lawson & Gemmell, 1990). Eggs are immediately infective when passed. Animals acquire infection from ingestion of food or water contaminated with sticky eggs, ingestion of segments or faeces containing eggs, or by eating arthropods such as dung beetles that carry eggs (Vargas-Calla *et al.*, 2018), though the latter is debated by Gonzales-Gustavson *et al.* (2023). Humans may be infected with *T. solium* by eggs on vegetables, in water, etc., that have been contaminated by faeces, or food contaminated by dirty hands, by faeco-oral transmission or possibly through retro-peristalsis and hatching of eggs internally (auto-infection). Disease Cysticercosis clusters where a human tapeworm carrier exists. Routine diagnosis of taeniosis continues to be mainly is based on the morphology of the adult tapeworm and the presence of eggs or segments in the faeces of infected definitive hosts.

Taenia spp. should be handled with appropriate biosafety and biocontainment measures 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

1. Identification of the agent

1.1. *Taenia saginata* (the beef tapeworm)

The adult is large, 4–8 metres long and can survive ~~(many) a number of~~ years, usually singly, in the small intestine of humans. The scolex (or head) has no rostellum or hooks. Useful morphological features are presented in Table 1 (~~Khalil et al., 1994; Loos-Frank, 2000; Soulsby, 1982; Verster, 1969~~). Gravid segments have >14 uterine branches. They usually leave the host singly and many migrate spontaneously from the anus. Tapeworm eggs are also released separately, outside the proglottid, in the faeces.

The eggs are typical 'taeniid' eggs that cannot be differentiated morphologically from other *Taenia* or *Echinococcus* spp. eggs. *Taeniid* eggs measure about 25–45 µm in diameter; contain an oncosphere (or hexacanth embryo) bearing three pairs of hooks; have a thick, brown, radially striated embryophore or 'shell' composed of blocks; and there is an outer, oval, membranous coat, the true egg shell, that is lost from faecal eggs.

Metacestodes of *T. saginata* usually occur in the striated muscles of cattle (beef measles), but also buffalo, and various *Cervidae*. Viable cysts are oval, fluid-filled, about 0.5–1 × 0.5 cm, translucent and contain a single white scolex that is morphologically similar to the scolex of the future adult tapeworm. They are contained in a thin, host-produced fibrous capsule. Cysts occasionally are found in the liver, lung, kidney, fat and elsewhere.

1.2. *Taenia solium* (the pork tapeworm)

Taenia solium is typically smaller than *T. saginata* being 1–5 metres and is generally assumed to survive for 2–3 years. The scolex has an armed rostellum bearing two rows of hooks. Gravid segments have <14 uterine branches and do not usually leave the host spontaneously, but passively (in small chains) with the faeces. Tapeworm eggs are also released separately, outside the proglottid, in the faeces.

Taenia solium cysticerci occur primarily in the muscles and central nervous system (CNS) of pigs (pork measles), and in the muscles, subcutaneous tissues, CNS and, rarely the eye, of humans. Cysts are grossly similar to those of *T. saginata*. They have a scolex bearing a rostellum and hooks similar to the adult. Occasionally, in the brain cisterns of humans, cysts can develop in the space available as racemose cysts up to 10 cm or more across that lack a scolex.

Table 1. Useful features for identification of scoleces and segments of *Taenia* spp.

Parasite species	Number of hooks	Length of hooks (µm)		No. testes	Layers of testes	Cirrus sac extends to longitudinal vessels	No. uterine branches	
		Large hooks	Small hooks					
<i>T. hydatigena</i>	28–36 (26–44)	191–218 (170–235)	118–143 (110–168)	600–700	1	Yes	6–10 that re-divide	Lobes of ovary unequal in size. No vaginal sphincter. Testes extend to vitellarium, but not confluent behind.
<i>T. ovis</i>	30–34 (24–38)	170–191 (131–202)	111–127 (89–157)	350–750	1	No	11–20 that re-divide	Lobes of ovary unequal in size. Well developed vaginal sphincter. Testes extend to posterior edge of ovary.

Parasite species	Number of hooks	Length of hooks (µm)		No. testes	Layers of testes	Cirrus sac extends to longitudinal vessels	No. uterine branches	
		Large hooks	Small hooks					
<i>T. multiceps</i>	22–30 (20–34)	157–177 (120–190)	98–136 (73–160)	284–388	2	Yes	14–20 that re-divide	Lobes of ovary equal in size. Pad of muscle on anterior wall of vagina. Testes extend to vitellarium, but not confluent behind.
<i>T. saginata</i>	– without rostellum	–	–	765–1200	1	No	14–32 that re-divide Ratio of uterine twigs to branches 2.3	Lobes of ovary unequal in size with small Well developed vaginal sphincter. Testes extend to vitellarium, but not confluent behind.
<i>T. solium</i>	22–36	139–200	93–159	375–575	1	Yes	7–14 that re-divide	Lobes of ovary unequal in size with small accessory lobe. No vaginal sphincter. Testes confluent behind vitellarium
<i>T. asiatica</i>	Vestigial hooks some with small rostellum	–	–	868–904		No	16–32 that re-divide Ratio of uterine twigs to branches 4.4	Ovary, vaginal sphincter and extent of testes as <i>T. saginata</i> . Posterior protuberances on some gravid segments

1.3. *Taenia asiatica* (Asian *Taenia*)

Closely related to but genetically distinguishable from *T. saginata*, the adult in humans has an ovary, vaginal sphincter muscle and cirrus sac like those of *T. saginata*, but *T. asiatica* has a small rostellum and posterior protuberances on some segments and 16–32 uterine buds with 57–99 uterine twigs on one side. Segments are passed singly and often spontaneously.

The metacestodes are small, about 2 mm, and have a rostellum and two rows of primitive hooks, those of the outer row being numerous and tiny. They occur mainly in the parenchyma and on the surface of the liver of domesticated and wild pigs; they may be found on the mesenteries and, rarely, are described in cattle, goats, and monkeys.

1.4. *Taenia ovis*

Adults in the intestine of dogs and wild canines reach 1–2 metres in length and have an armed rostellum; the number and size of hooks can aid differentiation of *Taenia* spp. (Table 1). Metacestodes that occur in the musculature (skeletal and cardiac) of sheep and less commonly goats reach 0.5–1.0 × 0.5 cm. A similar parasite (*T. ovis krabbei*) occurs in wild canines and dogs and the muscles of reindeer and deer in northern areas.

1.5. *Taenia hydatigena*

Adults are up to 1 metre or more long, are found in the intestine of dogs and wild canids, and have an armed rostellum (Table 1). Metacestodes can be large, from 1 cm up to 6–7 cm, and the scolex has a long neck. They are found attached to the omentum, mesentery and occasionally protruding from the liver surface, particularly of

sheep, but also of other domesticated and wild ruminants and pigs. A wolf and reindeer/deer cycle exists in northern latitudes, in which the metacestodes are found in the liver of the intermediate host; canids are definitive hosts.

1.6. *Taenia multiceps*

Adults, up to a metre long in the intestine of canids, have an armed rostellum (Table 1). The metacestodes (*Coenurus cerebralis*) are large, white fluid-filled cysts that may have up to several hundred scoleces invaginated on the wall in clusters. Coenuri grow to 5 cm or more in size in the brain of sheep, the brain and intermuscular tissues of goats, and also the brain of cattle, wild ruminants and occasionally humans. The cysts induce neurological signs that in sheep are called 'gid', 'sturdy', etc.

1.7. Diagnosis of adult parasites in humans or canine carnivores

All parasite or faecal material from humans with possible *T. solium* infections must be handled with suitable safety precautions to prevent accidental infection with the eggs. *Taenia multiceps* and *Echinococcus* spp. also infect humans and, as taeniid eggs in dogs cannot be differentiated to species or genus level, in areas where these are endemic, the same safety precautions apply. In addition to *Taenia* spp., humans and canine carnivores may be infected by ~~with~~ *Diphyllobothrium* and *Hymenolepis* spp., while six other cestode genera are recorded occasionally in humans. ~~These are described by Lloyd (2014)~~ and all can be differentiated from *Taenia* spp. by egg/proglottid morphology. In canids, *Echinococcus* spp. eggs cannot be distinguished from *Taenia* spp. eggs, but the presence of the former can be determined by tapeworm size and *Echinococcus*-specific antigen-capture enzyme-linked immunosorbent assay (Ag-ELISA) (Allan *et al.*, 1992). Other worms in canids, *Dipylidium*, *Diplopylidium*, *Mesocostoides* and *Diphyllobothrium* spp., have morphologically distinct eggs and proglottids (~~Lloyd, 2014; Soulsby, 1982~~).

Adult cestodes can be expelled from humans using an anthelmintic (praziquantel, niclosamide, albendazole) followed by a saline purgative and are identified on the basis of scolex and proglottid morphology, though scolices are often not recovered and comparison of proglottid morphology is not always a reliable method. A self-detection tool was used in Mexico (~~Flisser *et al.*, 2011~~); medical staff in health centres are supplied with preserved tapeworm segments in a bottle and a manual of questions to ask patients to try to identify carriers (no species identification). In animals, an anthelmintic, such as praziquantel, can be used as well; again, the recovered tapeworms are identified morphologically. Arecoline can no longer be recommended because of its side-effects. Tapeworms can be recovered after anthelmintic treatment, and require appropriate disposal.

~~Verster (1969) and Loos-Frank (2000) have given descriptions of described~~ parasitic diagnosis of all the *Taenia* spp. of humans and animals, their hosts and geographical distributions. ~~Keys for identification are given by Khalil *et al.* (1994).~~ Loos-Frank (2000) gives methods for mounting, embedding, sectioning and staining the proglottids. Worms, after relaxation in water, can be stained directly, although small worms should be fixed in ethanol for a few minutes. Alternatively, worms can be fixed and stored in 70% ethanol containing 10% lactic acid, the scolex and worm being stored separately. The rostellum, hooks and suckers of scoleces or protoscoleces should be cut off and mounted *en face* in Berlese's fluid (made by dissolving 15 g gum arabic in 20 ml distilled water and adding 10 ml glucose syrup and 5 ml acetic acid, the whole then being saturated with chloral hydrate, up to 100 g). The stain is lactic acid carmine: 0.3 g carmine is dissolved at boiling point in 42 ml lactic acid and 58 ml distilled water, 5 ml of 5% iron chloride solution ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$) is added after cooling and can be used again to refresh older solutions. Specimens are allowed to sink in the stain in a vial and are left in the stain for some more minutes to allow the stain to penetrate. Specimens are then washed in 1-day-old tap water until blue in colour. They are then fixed in 50–70% ethanol and dehydrated under the slight pressure of plastic foil keeping the segments flat. Salicylic acid methyl ester is used as clearant.

When segments break from the end of the worm, eggs are expelled in the intestine and can be found in the faeces. Segments of *T. saginata*, *T. asiatica* and the dog *Taenia* spp. may migrate spontaneously from the anus and this is likely to be noticed (>95% in the case of *T. saginata*). When the segments migrate, the sticky eggs are deposited in the perianal area and might be detected by application and examination of sticky tape. These signs are far less likely for *T. solium*. Segments of all three may be found on the faeces, but are passed intermittently. Even if a segment has shed all its eggs, it can be identified as a cestode by the many concentric calcareous corpuscles contained within its tissues. Faeces, after mixing to reduce aggregation, can be examined for eggs. Various techniques are used throughout the world and include ethyl acetate extraction and flotation. For the latter, NaNO_3 or Sheather's sugar solution (500 g sugar, 6.6 ml phenol, 360 ml water), with their higher specific gravities, are superior to saturated NaCl as flotation media for taeniid eggs. Flotation can be carried out in commercially marketed qualitative or quantitative flotation chambers (Mc Master technique, Mini-FLOTAC) or by centrifugal flotation that includes a modified Wisconsin technique (faeces, diluted in water, are sieved and centrifuged, the pellet is resuspended in sugar or Sheather's solution and centrifuged at 300 g for 4 minutes). Eggs adhering to the cover-slip can then be detected. Faecal egg examination will be less sensitive for *T. solium* than the other species. Species cannot be determined by egg morphology. Cheesbrough (2005; 2006) reports that *T. saginata*

eggs can be differentiated from *T. solium* on staining with Ziehl–Neelsen as used for acid-fast bacilli: the striated embryophore of *T. saginata* is acid fast (stains red), that of *T. solium* is not acid fast.

Molecular methods, including DNA probes, the polymerase chain reaction (PCR), PCR followed by restriction fragment length polymorphism (RFLP), or multiplex PCR, loop-mediated isothermal amplification (LAMP) or real-time PCR targeting cytochrome c oxidase subunit 1 (cox1), 12S, 18S and 28S (ribosomal genes) have proved useful for diagnosis (copro-PCR) and differentiation, though largely used experimentally, to differentiate faecal eggs of *T. solium*, *T. saginata* and *T. asiatica* (Gasser & Chilton, 1995; Geyson *et al.*, 2007; Gonzalez *et al.*, 2004; Ng-Nguyen *et al.*, 2017; Nkouawa *et al.*, 2009; Yamasaki *et al.*, 2004). While equally applicable to differentiation in dogs, the same examinations have not been done for *Taenia* spp.

Ag-ELISAs to detect *Taenia* coproantigen have been developed and can be established in-house if laboratory facilities are available (Allan *et al.*, 1992; ~~Deplazes *et al.*, 1994~~), with prior evaluation of appropriate controls. One Ag-ELISA was developed experimentally by Allan *et al.* (1992) to detect coproantigen in dogs, and so, with appropriate controls, could be used to detect *Taenia* infection in this species. The technique, however, is only *Taenia*-genus specific. A modification of the test, including polyclonal antibodies directed against excretory secretory antigens has been developed, rendering the test species specific (Guezala *et al.*, 2009). The test is an in-house, solid-phase, microwell assay with wells coated with polyclonal, rabbit anti-*Taenia*-specific antibody (TSA).

1.8. Diagnosis of metacestodes

Taenia solium metacestodes might be palpable in the tongue but, both in the living animal and on post-mortem examination or meat inspection, tongue palpation is of diagnostic value only in pigs heavily infected with metacestodes; these will also be difficult to differentiate from large sarcocysts, or other (mechanical) lesions. Nevertheless, tongue palpation is a common diagnostic method used by pig traders in endemic, resource poor areas. A sensitivity of 21% was determined by Dorny *et al.* (2004).

1.8.1. Meat inspection – the main diagnostic procedure

Metacestodes are visible first as very small, about 1 mm, cysts, but detection of these requires thin slicing of tissues in the laboratory. Many young cysts are surrounded by a layer or capsule of inflammatory cells (mononuclear cells and eosinophils being prominent histologically). The parasites' abilities to evade the immune response mean that later in infection, as the cyst matures, few inflammatory cells are present in its vicinity and the cysticercus in its intermuscular location is surrounded by a delicate fibrous tissue capsule.

In theory, cysts can be visualised or felt in tissues such as the tongue of heavily infected animals as early as 2 weeks after infection. Cysts are readily visible by 6 weeks and, when mature and viable, are usually oval, about 10 × 5 mm or larger (depending on the species), with a delicate, fairly translucent, white parasite membrane and host capsule. Pale fluid within the cyst and the scolex, visible as a white dot within the cyst, usually invaginates midway along the long axis of the cyst.

At meat inspection many of the *T. saginata* cysts detected, often as many as 85–100%, are dead. The rate at which cysts age and die and so degenerate varies with the parasite species and the tissue within which the cyst is embedded, the host's immunological status and possibly with host age at infection. In general, cysts tend to die more rapidly in the muscular predilection sites, such as the heart. The preferential distribution of parasites in these areas may be because of greater blood circulation to these muscles. Conversely, the higher rate of activity in these muscles may damage the parasites, allowing leakage of fluid and perhaps disrupting the parasites' abilities to evade the immune response. Cysts at different stages of viability and degeneration can be found in the same host. Death in skeletal muscles may occur within 2 months of infection of adult cattle with *T. saginata*, but cysts may remain viable for several years. Cysts of *T. hydatigena* in the peritoneal cavity of sheep and those of *T. solium* in the brain of pigs also have been described as surviving for long periods.

Degenerating cysts vary in appearance. Marked infiltration of eosinophils, macrophages, lymphocytes and collagen deposition thickens the capsule, which becomes opaque, but initially the cyst within remains apparently normal. The fluid gradually becomes colloid and inflammatory cells infiltrate. The cyst cavity becomes filled with greenish (eosinophilic) and then yellow, caseous material, usually being larger in size and certainly more obvious in meat than the original viable cyst. Later the cyst may calcify. Where very young (without a scolex) or degenerate cysts need to be differentiated from other lesions, compression of the cyst, smears of the caseous contents, histological examination of haematoxylin and eosin (H&E) stained sections are used. Microscopic examination may reveal the calcareous corpuscles (concentric concretions of salts that are around 5–10 µm in size). These indicate a cestode origin of the tissue. The presence of hooks and their length together with knowledge of the host and tissue may aid in identification of cestode species. Experimentally, immunohistochemical staining has differentiated *T. saginata* cysts

from non-*Taenia* structures. Molecular tools can be used as well, especially for species identification (see above), also in the finding of a new cestode in a host species or geographical area from which, historically, the parasite was absent. In one study PCR identified only 50% of degenerate presumed *T. saginata* cysts (Abuseir *et al.*, 2006), whereas Eichenberger *et al.* (2011) using different primers identified 80% of calcified lesions.

After treatment of *T. solium* in pigs with drugs such as oxfendazole (single dose, 30 mg/kg), the cysts may lose their fluid and collapse. The resultant lesion is smaller than lesions observed following natural death but can take 3–6 months to resolve.

Meat inspection procedures are defined, amongst others, by the regulations within a country or region (e.g. EU) and the animal species. Additional inspections can be performed on doubt or identification of a lesion, or specific origin of an animal. Examinations tend to be more extensive with the zoonotic ~~infections~~ cestodes *T. saginata* and *T. solium*.

In general, meat inspection procedures related to *Taenia* spp. consist of:

- i) Visual inspection of the carcass, its cut surfaces and the organs within it. This may reveal *T. saginata*, *T. solium* and *T. ovis* in the muscles, *T. hydatigena* on the liver (and *T. asiatica*), mesenteries or omentum, ~~or~~ and *T. multiceps* in the brain in the specific animal species.
- ii) The external and internal masseters and the pterygoid muscles are each examined and one or two incisions made into each, the cuts being parallel to the bone and right through the muscle.
- iii) The freed tongue is examined visually and palpated, particularly for *T. solium*.
- iv) The pericardium and heart are examined visually. The heart usually is incised once lengthwise through the left ventricle and interventricular septum so exposing the interior and cut surfaces for examination. Incisions may go from the base to the apex and regulations also may require additional, perhaps four, deep incisions into the left ventricle. Alternately, the heart may be examined externally and then internally after cutting through the interventricular septum and eversion.
- v) The muscles of the diaphragm, after removal of the peritoneum, are examined visually and may be incised.
- vi) The oesophagus is examined visually.
- vii) In some countries, the triceps brachii muscle of cattle is incised deeply some 5 cm above the elbow. Additional cuts into it may be made. The gracilis muscle also may be incised parallel to the pubic symphysis. These cuts are usually undertaken for *T. solium* in pigs. Such incisions into the legs are made, particularly in African countries as it is suspected that more parasites lodge in these muscles in working or range animals walking long distances because of the exercise and consequent increased blood flow to these muscles. Other countries may also require such incisions into the legs. However, as this devalues the meat, such incisions are made most commonly once one or more cysts have been found at the predilection sites so as to determine the extent of the infection.

Additional incisions may be required either by the regulations or if cysts are found on the initial incision(s). Eichenberger *et al.* (2013) reported an increase in sensitivity by multiple incisions, though this could not be confirmed by Jansen *et al.* (2018b). Details on meat inspection are supplied by Herenda *et al.* (2000).

Additional or fewer procedures may be required for specific parasites and the judgements on the carcass, viscera, offal and blood will vary dependent on *Taenia* spp. and regulations within a country.

1.8.2. Meat inspection – species differentiation and decision making

i) *Taenia saginata*: predilection sites

Calves under 6 weeks are not examined in certain countries (e.g. EU regulation). Predilection sites are the heart, tongue, masseters and diaphragm, presumably because they receive the greatest blood circulation. Nonetheless, cysts may be found in any muscle (or less frequently organs) of the body. Lesions of *T. saginata* may need to be differentiated from *Sarcocystis* sarcocysts and other lesions. In PCR studies in Germany, Switzerland and New Zealand up to 20% of viable, presumed *T. saginata* cysts could not be positively identified (Abuseir *et al.*, 2006). Meat inspection has a very low sensitivity (<16%) for the detection of *T. saginata*, especially with low infection levels (Dorny *et al.*, 2000; Eichenberger *et al.*, 2013; Kyvsgaard *et al.*, 1990). In general, meat inspection procedures detect only about 15–50% of the animals that are actually infected. Light infections are easily missed on palpation and meat inspection – in one study involving *T. saginata*, 78% of carcasses infected with >20 cysts were detected compared with those detected following dissection and slicing, while only 31% of those with fewer cysts were detected (Walther & Koske, 1980). Meat inspection efficacy

will vary with the number and location of incisions (and the skill and experience of the inspector). For example, in Zimbabwe, 58% of cattle were positive in the head only, 20% in the shoulder only and 8% in the heart only, although overall 81% were found to be infected if all three organs were included. In Kenya Walther & Koske (1980) also found that the predilection sites were not necessarily infected in 57% of the cattle found positive on dissection. They also confirmed the importance of the shoulder incisions in detection of infection in African countries as 20% of the cattle found to be infected were positive in the shoulder only. Animals infected as very young calves may have few or no cysts in tongue and masseters. Wanzala *et al.* (2003), also in Kenya, described the insensitivity of meat inspection in detecting cysticerci: only 50% of naturally or artificially infected cattle were identified. Their observations indicated that a number of viable cysticerci may be missed. A recent large-scale study on Belgian cattle, characterised by mostly light infections, even estimated the sensitivity as low as 0.54% (Jansen *et al.*, 2018a). While Eichenberger *et al.* (2011) detected substantially more cases when implementing additional incisions in the heart muscle (estimated sensitivity of 24.2%), this could not be confirmed by Jansen *et al.* (2018b) who defined a sensitivity of 2.87%.

ii) *Taenia solium*: predilection sites

The predilection sites of *T. solium* in pigs are as for *T. saginata* in cattle, although there are reports of higher prevalence in shoulder and thigh. Commonly one or more cuts are required 2.5 cm above the elbow joint. This is said to detect some 13% of infected carcasses that would otherwise have been missed. Incisions to be made are country regulation dependent. As for *T. saginata*, meat inspection has a low sensitivity (22.1%), especially for light infections (Dorny *et al.*, 2004). A recent study in South Africa found that the level of agreement (Kappa statistic) between carcass dissection (gold standard) and meat inspection was negative, which is an indication of disagreement between the two methods and confirms that the current meat inspection procedures alone are not sufficiently sensitive to detect all cases of porcine cysticercosis (Sithole *et al.*, 2019).

iii) *Taenia asiatica*: predilection sites

The small size means detection of cysts in the liver is difficult except in heavy infection.

iv) *Taenia hydatigena*: predilection sites

The parasite migrating in the liver leaves haemorrhagic tracks that then become green/brown with inflammation and later white due to fibrosis. For the records, these lesions must be differentiated from those of caused by liver flukes, if possible, by identification of the cysticerci or adult flukes. White spot from *Ascaris* infection is differentiated as the lesions appear as pale to white, small, isolated foci. Some cysts remain trapped below the liver capsule. These usually are small and degenerate early and then calcify into cauliflower-like lesions. Those that are retained at the liver surface are usually superficial and subserosal, while most of an *Echinococcus granulosus* hydatid cyst is deeper in the parenchyma. *Taenia hydatigena* cysts usually mature in the omental or mesenteric fat. If viable, the *T. hydatigena* cyst has a long-necked single scolex in virtually translucent cyst fluid. Fertile *Echinococcus* hydatid cysts have thicker walls and may contain many brood capsules containing protoscoleces; these appear as a sandy, whitish deposit within the cysts. Differentiation can be important in the implementation and monitoring of hydatid disease control measures so histology/molecular confirmation may be required. H&E-stained sections will reveal the laminated membrane of very young hydatid cysts ~~as indicated by Lloyd *et al.* (1991)~~. Its presence or absence can be confirmed by periodic acid–Schiff staining when the highly glycosylated proteins in the laminated membrane stain red. *Taenia hydatigena* lesions in cattle and pigs can be similar to tuberculosis. However, the portal and mesenteric lymph nodes are not involved, the contents of parasite cysts are more easily shelled-out and remainders of hooks and calcareous corpuscles may be seen or Ziehl–Neelsen staining may reveal bacteria.

v) *Taenia multiceps*: predilection sites

The parasites have a predilection ~~site~~ for the brain and spinal cord. Early migrating parasites can cause reddish haemorrhagic and later grey purulent tracks in the brain, and in heavy infections, the sheep may have a meningoencephalitis. Clinical signs caused by the mature cyst relate to pressure atrophy of adjacent nervous tissue and vary according to location in the brain. There may be impaired vision or locomotion if cysts are in the cerebral hemispheres and the sheep gradually may be unable to feed and will become emaciated. Cerebellar cysts may precipitate more acute and severe signs of ataxia or opisthotonus. In heavy infections, parasites migrate and begin development in other tissues, but they die early. These produce small lesions, 1 mm or so in size, that first contain an encapsulated cyst, then eosinophilic, caseous material that later may calcify. The site of *T. multiceps* cysts in sheep brain might be identified by clinical signs presented and, possibly, softening of the skull overlying the coenurus. Sometimes, the coenurus can be observed at the superficial surfaces of affected muscles, especially in the neck region.

vi) *Taenia ovis*: predilection sites

The predilection sites are as for *T. saginata*. Cysts may be confused with large *Sarcocystis gigantea* sarcocysts.

1.9. Detection of circulating antigens

The development of an automated sensitive and specific diagnostic test would greatly reduce the costs of damage to the carcass and also the costs of labour at meat inspection. Sensitivity of serological tests for animals has not reached the stage where commercialisation for individual diagnosis or large-scale detection of infected carcasses in slaughterhouses is possible. All assays tested – Ag-ELISA, antibody ELISA, enzyme-linked immunoelectro transfer blot (EITB) ~~and tongue inspection~~ – show low sensitivity in rural pigs infected naturally with low levels of *T. solium* (Dorny *et al.*, 2005; ~~Sciutto *et al.*, 1998~~). This finding is also true for *T. saginata* infections in cattle (Jansen *et al.*, 2018b; ~~Van Kerckhoven *et al.*, 1998~~). For example, only a small percentage (13–22%) of cattle carrying fewer than 30–50 viable cysticerci is detected by Ag-ELISA. Recent results estimate a sensitivity and specificity of 26.9 (increasing to 40% if only viable cysts are considered) and 99.4%, respectively for Ag ELISA in Belgian cattle (Jansen *et al.*, 2018b). Results of test performances can vary substantially between studies, representing different populations and study designs/analyses. In a Swiss study, a sensitivity and specificity of 14.3% (increasing to 40% if only viable cysts are considered) and 93.7%, were determined, respectively for the same Ag ELISA (Eichenberger *et al.*, 2013).

For the diagnosis of *T. solium* in pigs based on the B158/B60 Ag ELISA, for which a commercial kit is now available, latest results indicate a rather poor sensitivity and specificity (Chembensofu *et al.*, 2017; ~~Chilundo *et al.*, 2018~~; Kabululu *et al.*, 2020; Sithole *et al.*, 2019), though the sensitivity increases to 90% and 100% for the detection of carcasses with one or more viable cysticerci, and more than 10 viable cysts, respectively. The Ag ELISA was recently shown to have a low positive predictive value of 35.2 % (Kabululu *et al.*, 2020). As the test is genus specific, areas co-endemic with for example *T. hydatigena* complicate the usability of the test, which is particularly the case for Latin America and (South-East) Asia.

Nonetheless, Ag-ELISAs do have a use in field-based epidemiological studies for indicating transmission. The detection of viable infections in cattle or pigs could indicate point sources of infection, season of transmission and age of animals at risk. ~~The development of more sensitive and specific assays with recombinant antigens for diagnosis of NCC should improve immunodiagnosis of *T. solium* in pigs.~~

2. Serological tests for antibodies

Tests for circulating antibodies have little role in animals, except for epidemiological studies. A number of EITB and ELISAs for antibodies to *T. solium* in humans are now widely available. ~~These were reviewed by Rodriguez *et al.* (2012) with comparisons of sensitivity and specificity.~~ Recent Results indicate a sensitivity and specificity of 13.8% and 92.9%, respectively for antibody detection in Belgian cattle (Jansen *et al.*, 2018b), while the same test used in Swiss cattle in a different study, performed better with sensitivity and specificity of 81.6% and 96.3%, respectively (Eichenberger *et al.*, 2013).

In pigs, specific antibody detection, based on EITB and assuming the presence of one reactive band as a positive test result leads to a fairly good sensitivity of 89%, but a poor specificity of 48%. The latter can be improved by setting the cut-off at three bands needed for test positivity, leading to an increase in specificity reaching 76%, but with sensitivity decreasing to 78% (Jayashi *et al.*, 2013). Recent results suggest a cross-reactivity to GP50 in pigs infected with *Taenia hydatigena* (Muro *et al.*, 2017).

C. REQUIREMENTS FOR VACCINES

Effective vaccines have been developed against infection with the larval stages of several *Taenia* spp., but internationally accepted standards for the production of vaccines are not available. Considerable information is available in the scientific literature on immunogenic molecules, their recombinant technology and extraction, and their efficacy experimentally and in a number of field trials.

A recombinant vaccine based on the 45W antigen was developed in Australia and New Zealand against infection with *Taenia ovis* (~~Rickard *et al.*, 1995~~). The vaccine was approved for use in sheep in New Zealand but is not available commercially.

An effective recombinant vaccine has been developed against bovine cysticercosis based on the TSA9/18 antigens of *T. saginata* (Lightowlers *et al.*, 1996), but it has never been developed as a commercial product.

For *Taenia solium*, a vaccine based on the recombinant antigen TSOL18 (Flisser *et al.*, 2004) has been evaluated in independent experimental studies conducted in Mexico, Peru, Honduras and Cameroon in which the vaccine achieved 99–100% protection (Lightowlers, 2013). Subsequently, the vaccine has been used in the field in Nepal, Tanzania, Zambia,

Uganda, as well as in Peru where it was used in a project involving >55,000 pigs (Gabriel *et al.*, 2020; Garcia *et al.*, 2016; Kabululu *et al.*, 2020; Poudel *et al.*, 2019). The TSOL18 vaccine has been found to be both safe and efficacious. There is only one commercially available approved TSOL18 vaccine for pigs, produced in India. The TSOL18 vaccine targets the parasite during its early development in the pig, preventing infection. It does not affect cysticerci if they were already established in the tissues prior to vaccination. For that reason, use of the vaccine is recommended in combination with treatment using oxfendazole (single dose, 30 mg/kg), where the drug clears any pre-existing infection, while the vaccine prevents infection from any subsequent exposure to the parasite.

The commercial TSOL18 vaccine incorporates 150 µg of the TSOL18 antigen expressed in yeast, together with an oil adjuvant. Pigs of 2 months of age or older are immunised by intramuscular injection with 1 ml of the vaccine. A booster vaccination is required after 3–4 weeks. Booster vaccination may be applied after 6 months, however recent evidence suggests that pigs become naturally resistant to new *T. solium* infections as they age (Poudel *et al.*, 2019), suggesting that booster vaccination of animals fully vaccinated as young animals may not be required. Generally, no significant side effects are noticed, however in some animals, temporary pyrexia, lethargy for 1–2 days and local injection site reactions for up to 7 days may be observed after vaccination. Following administration of a 5-fold overdose of vaccine, no adverse reactions were observed under farm conditions other than those described above. The vaccine should be stored and transported between 2°C and 8°C; it should not be frozen. Although the manufacturer recommends the vaccine be maintained refrigerated, being a defined antigen, non-living vaccine, the vaccine is known to be relatively insensitive to exposure to room temperatures.

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NB: At the time of publication (2021) there were no WOA Reference Laboratories for cysticercosis (including infection with *Taenia solium*) (please consult the WOA Web site: <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>).

NB: FIRST ADOPTED IN 1991. MOST RECENT UPDATES ADOPTED IN 2021.

Annex 21. Collaborating Centres Satisfaction Questionnaire

MEETING OF THE BIOLOGICAL STANDARDS COMMISSION

Paris, 8–12 September 2025



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