91st General Session
World Organisation for Animal Health
World Assembly
Paris, 26-30 May 2024
GS

91 GS/Tech-07/En
Original: English
May 2024

Activities of the Specialist Commissions

BIOLOGICAL STANDARDS COMMISSION

Proposed amendments to the
Manual of Diagnostic Tests and Vaccines for Terrestrial Animals

Technical Working Document

World Organisation for Animal Health
Founded as OIE

Contents

Annex numbers correspond to the number of the annexes in the report of the February 2024 meeting of the Biological Standards Commission

I. Introduction

Since the 90th General Session in May 2023, the Biological Standards Commission met twice, from 4 to 8 September 2023 and from 5 to 9 February 2024. Among its activities, the Commission progressed its work on the development of new and revised texts for the *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (*Terrestrial Manual*) in accordance with its work programme. Details of the Commission's activities, including the texts circulated for comment, were published in the Commission's September 2023 and February 2024 meeting reports and are available on the Delegates' website as well as the WOAH Website.

This report provides a brief summary of each of the revised texts of the *Terrestrial Manual* that will be presented for adoption at the 91st General Session. Details of the Commission's consideration of Member comments received on draft texts, were provided in the Commission's September 2023 and February 2024 meeting reports. The Commission encourages Members to refer to these meeting reports for more details about the amended texts to be proposed for adoption.

The annexes to this document present the proposed amendments to the chapters from the *Terrestrial Manual* that will be presented to the World Assembly of Delegates for adoption at the 91st General Session. The annex numbers align with the annex numbers provided in the Biological Standards Commission's February 2024 report.

Further amendments to the draft text may be proposed during the General Session based on the Member comments received during the second round of comments (deadline 30 April 2024).

In the process of drafting and reviewing these amendments, the Commission considered comments submitted by Members and by International Organisations that have a cooperation agreement with WOAH.

1. *Terrestrial Manual* texts to be proposed for adoption

1.1 Chapter 1.1.5. 'Quality management in veterinary testing laboratories' (Annex 4)

Chapter 1.1.5. 'Quality management in veterinary testing laboratories' has undergone an extensive revision. The revised text was first circulated for comment in October 2023. The main amendments include: updated the references and URLs; clarified that limited availability of suitable material may render validation difficult and moved to Section A.7.3 'Validation of the test method'; included substantial technical updates to the sections on:

- Accreditation;
- Determination of the scope of the quality management system or of the laboratory's accreditation;
- Validation of the test method;
- Estimation of measurement uncertainty.

And updated the section on strategic planning.

1.2 Chapter 1.1.9. 'Tests for sterility and freedom from contamination of biological materials intended for veterinary use' (Annex 5)

Chapter 1.1.9. 'Tests for sterility and freedom from contamination of biological materials intended for veterinary use' has undergone an extensive revision. The revised text was first circulated for comment in October 2023. The main amendments include: updated to give an example-based overview of tests and their regulatory background including brief illustrative examples of vaccine contamination; added more details to Section G. 'Protocol examples' and clearly marked the examples as non-prescriptive and non-exhaustive – they are a strong motivator for extraneous agent testing; updated Section A. 'An overview of testing approaches' with more recent opportunities and challenges; merged sections on living and inactivated viruses and bacteria, to simplify and streamline the chapter; updated references and weblinks.

1.3 Chapter 2.2.4. 'Measurement uncertainty' (Annex 6)

Chapter 2.2.4. 'Measurement uncertainty' has undergone an extensive revision. The revised text was first circulated for comment in October 2023. The main amendments include: removed reference to the 'WOAH Validation Standard': Chapter 1.1.6 will differ from the future one in the *Aquatic Manual*, so there is no longer one Standard that applies to both Manuals; clarified that the method described in the chapter is known as the 'top-down' approach, and included information of its requirements along with a section on the scope and limitations of the top-down approach; clarified that alternative methods are available that are less reliant on distributional assumptions, and better handle the presence of outliers; added an example of a measurement uncertainty calculation in molecular tests.

1.4 Chapter 2.2.6. 'Selection and use of reference samples and panels' (Annex 7)

Chapter 2.2.6. 'Selection and use of reference samples and panels' has undergone a moderate revision. The revised text was first circulated for comment in October 2023. The main amendments include: updated cross-references to Chapter 1.1.6 'Validation of diagnostic assays for infectious diseases of terrestrial animal'; added a figure on the documentation that is required of reference materials; added a list of references for further reading; added a section on 'Further reading' with peer-reviewed references.

1.5 Chapter 3.1.5. 'Crimean–Congo haemorrhagic fever' (Annex 8)

Chapter 3.1.5. 'Crimean–Congo haemorrhagic fever' has undergone a moderate revision. The revised text was first circulated for comment in October 2023. The main amendments include: added two footnotes to the purpose 'Confirmation of clinical cases in animals' in Table 1 'Diagnostic test formats for Crimean–Congo haemorrhagic fever virus infections in animals' to ensure that it aligns with the case definition: the footnotes will be replaced with a link to the case definition once it is adopted and included in the *Terrestrial Code;* changed the rating of the real-time PCR for the purpose 'Individual animal freedom from infection prior to movement' as research into CCHV confirms transient viremia.

1.6 Chapter 3.3.6. 'Avian tuberculosis' (Annex 9)

Chapter 3.3.6. 'Avian tuberculosis' has undergone an extensive revision. The revised text was first circulated for comment in October 2023. The main amendments include: updated the nomenclature and classification of species in the genus *Mycobacterium;* reviewed the ratings of some of the tests in Table 1 'Test methods available for the diagnosis of avian tuberculosis and their purpose'; updated the section on nucleic acid recognition methods; added a section on the stained antigen test; updated the section on the production and minimum requirements for tuberculin production; updated the list of references. **NB:** as avian TB is not a listed disease, this chapter will be removed from the *Terrestrial Manual;* once adopted, the information on avian tuberculin production will be moved to Chapter 3.1.13 'Mammalian tuberculosis (infection with *Mycobacterium tuberculosis* complex)'.

1.7 Chapter 3.4.1. 'Bovine anaplasmosis' (Annex 10)

Chapter 3.4.1. 'Bovine anaplasmosis' has undergone an extensive revision. The revised text was first circulated for comment in October 2023. The main amendments include: updated the information in the introduction to the chapter; added an illustration of a stained blood smear showing *Anaplasma marginale* inclusion bodies; thoroughly updated the section on the PCR, including adding a table of primer sequences, and the section on the ELISAs including adding a displacement double-antigen sandwich ELISA that has been developed to differentiate between *A. marginale* and *A. centrale* antibodies; reviewed the ratings of some of the tests in Table 1 'Test methods available for the diagnosis of bovine anaplasmosis and their purpose'; emphasised that the complement fixation test has variable sensitivity and removed it from Table 1. The chapter now includes justification tables for the ratings of the tests in Table 1 for each purpose. These justification tables will be extremely useful to users of the *Terrestrial Manual* when deciding which test to use for a given purpose.

1.8 Chapter 3.4.7. 'Bovine viral diarrhoea' (*Annex 11*)

Chapter 3.4.7. 'Bovine viral diarrhoea' has undergone a moderate revision. The revised text was first circulated for comment in October 2023. The main amendments include: updated the taxonomy; reviewed the ratings of some of the tests in Table 1 'Test methods available for the diagnosis of bovine viral diarrhoea and their purpose'; developed justification tables for the ratings of the tests in Table 1 for each purpose.

1.9 Chapter 3.4.12. 'Lumpy skin disease' (*vaccine section only*) (*Annex 12*)

Chapter 3.4.12. 'Lumpy skin disease' (vaccine section only) has undergone an extensive revision. The revised text was first circulated for comment in October 2023. The main amendments include: added text on the scarcity of information on the role of wildlife in lumpy skin disease (LSD) epidemiology; thoroughly updated Section C 'Requirements for vaccines'.

1.10 Chapter 3.6.9. 'Equine rhinopneumonitis (*infection with Varicellovirus equidalpha1*)*'* [*formally equid herpesvirus-1*] (*Annex 13*)

Chapter 3.6.9. 'Equine rhinopneumonitis (infection with Varicellovirus equidalpha1' [formally equid herpesvirus-1]) has undergone an extensive revision. The revised text was first circulated for comment in October 2023. The main amendments include: updated the taxonomy of the pathogenic agent: equid herpesvirus-1 is now Varicellovirus equidalpha1 – as the chapter covers infection with Varicellovirus equidalpha1, most of the information on equid herspsvirus-4 has been removed as EHV4 is not listed; thoroughly updated Section B 'Diagnostic techniques' in particular the section on virus detection by PCR, which now has a table on primer and probe sequences for a number of real-time PCRs and subsections on POC molecular tests and molecular characterisation, and the sections on virus isolation and on virus neutralisation, and added a section on the complement fixation test; developed justification tables for the ratings of the tests in Table 1 for each purpose.

1.11 Chapter 3.8.1. 'Border disease' (*Annex 14*)

Chapter 3.8.1. 'Border disease' has undergone a minimal revision. The revised text was first circulated for comment in October 2023. The main amendments include: minor update, mainly of the taxonomy.

1.12 Chapter 3.8.12. 'Sheep pox and goat pox' (*diagnostic test section only*) (*Annex 15*)

Chapter 3.8.12. 'Sheep pox and goat pox' (diagnostic test section only) has undergone a moderate revision. The revised text was first circulated for comment in October 2023. The main amendments include: included fluorescent antibody testing histopathology and ELISA in Table 1 'Test methods available for diagnosis of sheep pox and goat pox and their purpose'; thoroughly updated the section on nucleic acid recognition methods, in particular the conventional and real-time PCR methods; clarified that ELISAs cannot discriminate between antibodies to different capripoxviruses.

1.13 Chapter 3.9.1. 'African swine fever' (*vaccine section only*) (*Annex 16*)

Chapter 3.9.1. African swine fever (vaccine section only) has undergone an extensive revision. The revised text was first circulated for comment in October 2023. The main amendments include: updated Section C 'Requirements for vaccines' on the manufacture of pure, potent, safe and efficacious vaccines for ASF including key vaccine performance and quality criteria after consultation with vaccine developers, subject matter experts, representatives from the scientific community, regulatory authorities and WOAH Reference Laboratories. An appendix to the draft chapter was also included for information in the September 2023 report comprising the results of the consultation, the key parameters, summaries of discussions, scientific justifications, etc.

Annex 4: Chapter 1.1.5. 'Quality management in veterinary testing laboratories'

CHAPTER 1.1.5.

QUALITY MANAGEMENT IN VETERINARY TESTING LABORATORIES

SUMMARY

Valid laboratory results are essential for diagnosis, surveillance, and trade. Such results are ~~achieved by the use~~ assured through implementation of ~~good~~ a management ~~practices, valid~~ system that supports accurate and consistent test and calibration methods~~, proper techniques, quality control and quality assurance, all working together within a quality management system~~. Laboratory quality management includes technical, managerial, and operational elements of ~~testing~~ performing, interpreting and ~~the interpretation of~~ reporting test results. A quality management system enables the laboratory to demonstrate both competency and an ability to generate consistent technically valid results that meet the needs of its customers. ~~The need for~~ Mutual recognition and acceptance of test results for international trade, and the ~~acceptance~~ accreditation of tests to international standards such as ISO/IEC[1] 17025~~:2005~~ (General Requirements for the Competence of Testing and Calibration Laboratories) (ISO/IEC, ~~2005~~ 2017b) requires ~~good~~ suitable laboratory quality management systems. This chapter is not intended to reiterate the requirements of ISO/IEC 17025, nor has it been endorsed by accreditation bodies. Rather, it outlines the important issues and considerations a laboratory should address in the design and maintenance of its quality management system, ~~whether or not it has been formally accredited~~ regardless of formal accreditation status. Chapter 1.1.1 Management of ~~veterinary diagnostic laboratories *gives an introduction to*~~ veterinary diagnostic laboratories *introduces the components of governance and management of veterinary laboratories that are necessary for the effective delivery of diagnostic services, and highlights the critical elements that should be established as minimum requirements.*

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

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

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

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

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

i) Type of testing ~~done~~ performed, e.g. research versus diagnostic work;

ii) Purpose and requirements of ~~the~~ test results, e.g. ~~for~~ import ~~or~~ /export quarantine testing, surveillance, emergency disease exclusion, declaration of freedom from disease post-outbreak;

iii) Potential impact of a questionable ~~or~~, erroneous or unfavourable result, e.g. detection of foot and mouth disease (FMD) in an FMD-free country;

iv) ~~The tolerance level of~~ Risk and liability tolerance, e.g. vaccination ~~vs~~ versus culling ~~or~~ /slaughter;

v) Customer ~~needs~~ (requirements, e.g. sensitivity and specificity ~~of the test method~~, cost, turnaround time, ~~strain or genotype~~ level of characterisation~~), e.g. for surveillance, or declaration of freedom after outbreak~~;

vi) ~~The role of the laboratory~~ Role in legal work or in regulatory programmes, e.g. for disease eradication and declaration of disease freedom to the WOAH;

vii) ~~The role of the laboratory~~ Role in assisting with, confirming, or overseeing the work of other laboratories (e.g. as a reference laboratory);

viii) Business goals ~~of the laboratory~~, including the need for any third-party recognition or accreditation.

2. Standards, guides, and references

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

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

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

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

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

3. Accreditation

If ~~the laboratory decides to proceed with~~ formal recognition of ~~its~~ a laboratory's quality management system and testing~~, then~~ is sought, third party verification of its conformity with the selected standard(s) ~~will be~~ is necessary. ILAC has published specific requirements and guides for laboratories and accreditation bodies. Under the ILAC system, ISO/IEC 17025 is to be used for laboratory accreditation of testing or calibration activities. Definitions regarding laboratory accreditation may be found in ISO/IEC International Standard 17000: Conformity Assessment – Vocabulary and General Principles (ISO/IEC, ~~2004a~~ 2020). Accreditation is ~~tied to~~ dependent on demonstrated competence, which ~~is~~ encompasses significantly more than having and following documented procedures. Providing a competent and customer-oriented service also ~~means that the laboratory~~ requires:

i) Adequate facilities and environmental controls;

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

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

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

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

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

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

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

iv) Appropriate sample and materials management processes;

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

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

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

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

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

x) Complaints management;

xi) Adequate control of data and information;

xii) Appropriate reporting and approval process;

xiii) Culture of continual improvement.

xiv) Has demonstrable competence to generate technically valid results.

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

4. Selection of an accreditation body

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

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

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

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

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

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

i) Associated risks and opportunities;

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

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

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

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

vi) Availability of personnel, facilities and equipment;

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

viii) Access to proficiency testing schemes;

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

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

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

6. Quality assurance, quality control and proficiency testing

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

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

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

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

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

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

7. Test methods

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

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

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

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

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

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

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

7.1. Selection of the test method

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

7.1.1. Considerations for the selection of a test method

i) International acceptance;

ii) Scientific acceptance;

iii) Appropriate or current technology;

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

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

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

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

viii) Test turnaround time;

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

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

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

xii) Customer expectations;

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

xiv) Cost of test, per sample;

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

7.2. Optimisation and standardisation of the test method

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

7.2.1. Determinants of optimisation

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

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

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

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

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

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

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

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

7.3. Validation of the test method

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

7.3.1. Activities that validation might include

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

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

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

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

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

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

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

v~~iii~~) Analysis of internal quality control data.

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

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

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

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

~~7.4. Uncertainty of the test method~~

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

7.4. Estimation of Measurement Uncertainty

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

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

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

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

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

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

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

7.4.1. Potential sources of uncertainty include:

i) Sampling;

ii) Contamination;

iii) Sample transport and storage conditions;

iv) Sample processing;

v) Reagent quality, preparation and storage;

vi) Type of reference material;

vii) Volumetric and weight manipulations;

viii) Environmental conditions;

ix) Equipment effects;

x) Analyst or operator bias;

xi) Biological variability;

xii) Unknown or random effects.

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

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

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

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

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

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

~~i) Sampling;~~

~~ii) Contamination;~~

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

iv) ~~Sample processing;~~

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

vi) ~~Type of reference material;~~

vii) ~~Volumetric and weight manipulations;~~

viii) ~~Environmental conditions;~~

ix) ~~Equipment effects;~~

x) ~~Analyst or operator bias;~~

xi) ~~Biological variability;~~

xii) ~~Unknown or random effects.~~

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

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

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

7.5. Implementation and use of the test method

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

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

8. Strategic planning

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

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

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

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

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

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

vi) Conducting research;

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

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

ix) Planned, continual professional development and technical training;

x) Management reviews;

xi) Analysis of customer feedback;

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

REFERENCES

ANSI (American National Standards Institute) (1997). ANSI/NCS[5] Z540-2-1997 (R2012), US Guide to the Expression of Uncertainty in Measurement, First Edition. American National Standards Institute, www.ansi.org.

Colling A.& Gardner I.A., eds (2021). Diagnostic test validation science: a key element for effective detection and control of infectious animal diseases. *Rev. Sci. Tech. Off. Int. Epiz.,* **40**, Available at https://doc.woah.org/dyn/portal/index.xhtml?page=alo&aloId=41245

Eurachem (2007). Eurachem/CITAC Guide: Use of Uncertainty Information in Compliance Assessment, First Edition. Eurachem Secretariat. www.eurachem.org.

Eurachem (2012). Eurachem/CITAC[6] Guide (CG4): Quantifying Uncertainty in Analytical Measurement, Third Edition. Eurachem Secretariat. www.eurachem.org.

ISO (2015). ISO 9001:2015. Quality Management Systems – Requirements. International Organization for Standardization (ISO), www.iso.org.

ISO/IEC (~~2004a~~ 2020). ISO/IEC 17000:~~2004~~ 2020. Conformity Assessment – Vocabulary and General Principles. International Organization for Standardization (ISO), www.iso.org.

ISO/IEC (~~2004b~~ 2017a). ISO/IEC 17011:~~2004~~2 017. Conformity Assessment – General Requirements for accreditation Bodies Accrediting Conformity Assessment Bodies. International Organization for Standardization (ISO), www.iso.org.

ISO/IEC (~~2005~~ 2017b). ISO/IEC 17025:~~2005~~ 2017. General Requirements for the Competence of Testing and Calibration Laboratories. International Organization for Standardization (ISO), www.iso.org.

ISO/IEC (2010). ISO/IEC 17043:2010. Conformity Assessment – General Requirements for Proficiency Testing. International Organization for Standardization (ISO), www.iso.org

ISO/IEC (2022 ~~2012~~). ISO/IEC 15189:~~2012~~ 2022. Medical Laboratories – Requirements for Quality and Competence. International Organization for Standardization (ISO), www.iso.org

[5] NCSL: The National Conference of Standards Laboratories.
[6] CITAC: The Cooperation of International Traceability in Analytical Chemistry.

Johnson P. & Cabuang L. (2021). Proficiency testing and ring trials. *Rev. Sci. Tech. Off. Int. Epiz.*, **40**, 189–203.

Newberry K. & Colling A. (2021). Quality standards and guidelines for test validation for infectious diseases in veterinary laboratories. *Rev. Sci. Tech. Off. Int. Epiz.*, **40**, 227–237.

STEVENSON M., HALPIN K. & HEUER C. (2021). Emerging and endemic zoonotic diseases: surveillance and diagnostics. *Rev. Sci. Tech. Off. Int. Epiz.*, **40**, 119–129.

Waugh C. & Clark G. (2021). Factors affecting test reproducibility among laboratories. *Rev. Sci. Tech. Off. Int. Epiz.*, **40**, 131–141.

*

* *

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

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

CHAPTER 1.1.9.

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

INTRODUCTION

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

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

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

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

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

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

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

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

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

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

A. AN OVERVIEW OF TESTING APPROACHES

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

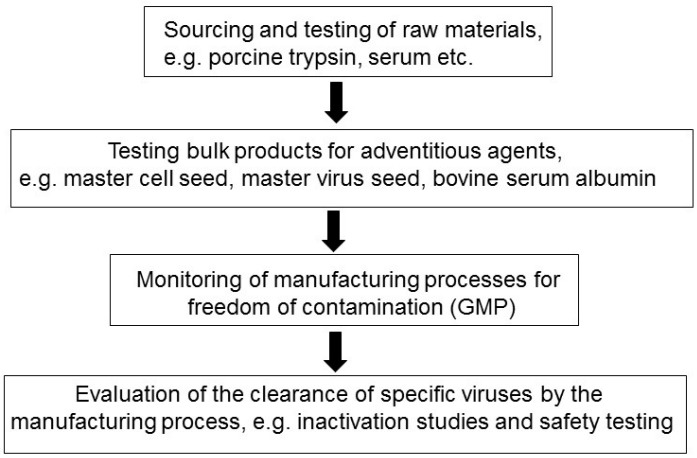
- Primary materials must be collected from sources shown to be free from contamination and handled in such a way as to minimise contamination and the opportunities for any contaminants to multiply (Figure 1).

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

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

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

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



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

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

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

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

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

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

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

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

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

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

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

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

~~1. Section B applies.~~

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

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

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

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

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

~~E.~~ D. LIVING BACTERIAL VACCINES

1. See Section B ~~applies~~.

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

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

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

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

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

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

~~F. INACTIVATED BACTERIAL VACCINES~~

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

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

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

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

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

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

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

~~H.~~ F. EMBRYOS, OVA, SEMEN

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

~~J.~~ G. PROTOCOL EXAMPLES

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

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

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

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

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

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

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

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

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

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

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

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

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

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

2.1.1. Diluent A

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

2.1.2. Diluent B

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

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

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

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

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

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

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

2.1.3. Example of growth promotion and test interference

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

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

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

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

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

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

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

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

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

2.3~~2~~. Example of general procedure for testing seed lots of bacteria and live bacterial biologicals for purity

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

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

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

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

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

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

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

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

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

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

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

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

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

3. Example of detection of *Mycoplasma* contamination

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

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

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

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

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

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

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

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

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

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

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

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

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

3.2 General testing for exclusion of *Mycoplasma* sp.

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

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

and

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

4. Example of detection of rickettsia and protozoa

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

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

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

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

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

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

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

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

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

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

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

Cell seed stocks do not require a neutralisation process.

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

5.1.1 Example of amplification in cell culture

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

5.1.2 Example of general detection procedures: cytopathology

May–Grünwald–Giemsa or H&E staining procedures are used to assess for cytopathological changes associated with virus growth. Monolayers must have a surface area of at least 6 cm^2 and can be prepared on appropriate chambered tissue culture slides and incubated for 7 days. The plastic wells of the slides are removed leaving the rubber gasket attached to the slide.

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

5.1.3 Example of general detection procedures: haemadsorption

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

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

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

5.2.1. Example of porcine epidemic diarrhoea virus (PEDV)

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

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

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

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

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

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

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

- European Commission (2015). The Rules Governing Medicinal Products in the European Union. Eudralex. Volume 6. Notice to applicants and regulatory guidelines for medicinal products for veterinary use.

- Department of Agriculture, Forest and Fisheries of Australia (2021b). Live veterinary vaccines Summary of information required for biosecurity risk assessment, Version 6 and Inactivated veterinary vaccines, Version 8.

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

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

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

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

I. RISK ANALYSIS PROCESS

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

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

~~I~~ J. BIOCONTAINMENT

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

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

REFERENCES

ALHASSAN A., GOVIND Y., TAM N.T., THEKISOE O.M., YOKOYAMA N., INOUE N. & IGARASHI I. (2007). Comparative evaluation of the sensitivity of LAMP, PCR and *in vitro* culture methods for the diagnosis of equine piroplasmosis. *Parasitol. Res*., **100**, 1165–1168.

AUSTRALIAN QUARANTINE POLICY AND REQUIREMENTS FOR THE IMPORTATION OF LIVE AND NOVEL VETERINARY BULK AND FINISHED VACCINES (1999). Available online at: https://www.agriculture.gov.au/sites/default/files/sitecollectiondocuments/ba/memos/1999/animal/99-085acleaned.pdf (Accessed 25 July 2023).

AVARZED A., IGARASHI I., KANEMARU T., HIRUMI, K., OMATA T., SAITOY., OYAMADA A., NAGASAWA H., TOYODA Y. & SUZUKI N. (1997). Improved *in vitro* cultivation of *Babesia caballi*. *J. Vet. Med. Sci*., **59**, 479–481.

BARONE P., WIEBE M.E., LEUNG J.C., HUSSEIN I.T.M., KEUMURIAN F.J., BOURESSA J., BRUSSEL A., CHEN D., CHONG M., DEHGHANI H., GERENTES L., GILBERT J., GOLD D., KISS R., KREIL T.R., LABATUT R.,. LI Y, MÜLLBERG J., MALLET L., MENZEL C., MOODY M., MONPOEHO S., MURPHY M., PLAVSIC M., ROTH N.J., ROUSH D., RUFFING

M., SCHICHO R., SNYDER R., STARK D., ZHANG C., WOLFRUM J., SINSKEY A.J. & SPRINGS S.L. (2020). Viral contamination in biologic manufacture and implications for emerging therapies. *Nat. Biotechnol.,* **38**, 563–572.

BAYLIS S.A., FINSTERBUSCH T., BANNERT N., BLUMEL J. & MANKERTZ A. (2011). Analysis of porcine circovirus type 1 detected in Rotarix vaccine. *Vaccine*, **29**, 690–697.

CODE OF FEDERAL REGULATIONS (OF THE UNITED STATES OF AMERICA) (CFR) (2015). Subchapter E. Viruses, serums, toxins and analogous products; organisms and vectors. *In*: Code of Federal Regulations, Animals and Animal Products. Title 9, Parts 101–124. US Government Printing Office, Washington DC, USA. Available online; https://www.law.cornell.edu/cfr/text/9/part-113 (Accessed 25 July 2023).

DEPARTMENT OF AGRICULTURE, WATER AND ENVIRONMENT (OF AUSTRALIA) (2013). Review of Published Tests to Detect Pathogens in Veterinary Vaccines Intended for Importation into Australia, Second Edition. CC BY 3.0. Available online at: https://www.agriculture.gov.au/biosecurity-trade/policy/risk-analysis/animal/review-of-published-tests-to-detect-pathogens (Accessed 25 July 2023).

DEPARTMENT OF AGRICULTURE, WATER AND ENVIRONMENT (OF AUSTRALIA) (2021a). Inactivated veterinary vaccines Summary of information required for biosecurity risk assessment. Version 8. Available online at: https://www.agriculture.gov.au/sites/default/files/sitecollectiondocuments/aqis/importing/bio-products/vet-vaccines/inact-vet-vacs.pdf (Accessed 25 July 2023).

DEPARTMENT OF AGRICULTURE, WATER AND ENVIRONMENT (OF AUSTRALIA) (2021b). Live veterinary vaccines. Summary of information required for biosecurity risk assessment. Version 6. Available online at: https://www.awe.gov.au/sites/default/files/sitecollectiondocuments/aqis/importing/bio-products/vet-vaccines/live-vet-vaccines.doc (Accessed 25 July 2023).

EUROPEAN COMMISSION (~~2006–~~2015). The Rules Governing Medicinal Products in the European Union. Eudralex. Volumes 4–9. European Commission, DG Health and Food Safety, Public health, EU Pharmaceutical information, Eudralex. Available online at: http://ec.europa.eu/health/documents/eudralex/index_en.htm (accessed 25 July 2023).

EUROPEAN MEDICINES AGENCY SCIENCES MEDICINES HEALTH (2016). EMA/CVMP/IWP/251741/2015. Reflection paper on methods found suitable within the EU for demonstrating freedom from extraneous agents of the seeds used for the production of immunological veterinary medicinal products. Available online at: https://www.edqm.eu/en/d/95422?p_l_back_url=%2Fen%2Fsearch-edqm%3Fq%3DEMA%252FCVMP%252FIWP%252F251741%252F2015%26delta%3D40%26category%3D302281 (Accessed 25 July 2023).

EUROPEAN PHARMACOPOEIA ~~8.2. (2014) (2021–~~2023). European Directorate for the Quality of Medicines and Health Care (EDQM), Council of Europe, Strasbourg, France. ~~Available online at http://online.edqm.eu/ (accessed 8 March 2017) Current version available online at European Pharmacopoeia (Ph. Eur.) 10th Edition – European Directorate for the Quality of Medicines & HealthCare (edqm.eu) (accessed 4 July 2022)~~ Current version available online at European Pharmacopoeia (Ph. Eur.) 11th Edition – European Directorate for the Quality of Medicines & HealthCare (edqm.eu) (accessed 25 July 2023).

FARSANG A. & KULCSAR G. (2012). Extraneous agent detection in vaccines – a review of technical aspects. *Biologicals*, **40**, 225–230.

HARE W.C.D. (1985). Diseases Transmissible by Semen and Embryo Transfer Techniques. OIE Technical Series No. 4. World Organisation for Animal Health (OIE), Paris, France.

HODINKA R.L. (2013). Point: is the era of viral culture over in the clinical microbiology laboratory? *J. Clin. Microbiol*., **51**, 2–4.

IKADAI H., MARTIN M.D., NAGASAWA H., FUJISAKI K., SUZUKI N., MIKAMI T., KUDO N., OYAMADA T. & IGARASHI I. (2001). Analysis of a growth-promoting factor for *Babesia caballi* cultivation. *J. Parasitol*., **87**, 1484–1486.

MARCUS-SECURA C., RICHARDSON J.C., HARSTON R.K., SANE N. & SHEETS R.L. (2011). Evaluation of the human host range of bovine and porcine viruses that may contaminate bovine serum and porcine trypsin used in the manufacture of biological products. *Biologicals*, **39**, 359–369.

MACKAY D. & KRIZ N. (2010). Current challenges in viral safety and extraneous agents testing. *Biologicals*, **38**, 335–337.

MILLER P.J., AFONSO C.L., SPACKMAN E., SCOTT M.A., PEDERSEN J.C., SENNE D.A., BROWN J.D., FULLER C.M., UHART M.M., KARESH W.B., BROWN I.H., ALEXANDER D.J. & SWAYNE D. (2010). Evidence for a new avian paramyxovirus serotype 10 detected in rockhopper penguins from the Falkland Islands. *J. Virol.,* **84**, 11496–11504.

NEVEROV A. & CHUMAKOV K. (2010). Massively parallel sequencing for monitoring genetic consistency and quality control of live viral vaccines. *Proc. Natl Acad. Sci.* USA, **107**, 20063–20068.

ONIONS D. & KOLMAN J. (2010). Massively parallel sequencing, a new method for detecting adventitious agents. *Biologicals*, **38**, 377–380.

OUTLINE OF THE REGULATORY SYSTEM OF VETERINARY DRUGS IN JAPAN (2015). Assurance of the Quality, Efficacy, and Safety Based on the Law for Ensuring the Quality, Efficacy, and Safety of Drugs and Medical Devices. https://www.maff.go.jp/nval/english/regulatory/pdf/Outline_RegulatorySystem_VMPs.pdf (Accessed 25 July 2023).

ROSSEEL T., LAMBRECHT B., VANDENBUSSCHE F., VAN DEN BERG T. & VAN BORM S. (2011). Identification and complete genome sequencing of paramyxoviruses in mallard ducks (*Anas platyrhynchos*) using random access amplification and next generation sequencing technologies. *Virol. J.*, **8**, 463.

SHEETS R., LOEWER, J., RAYDCHAUDHURI G. & PETRICCIANI J. (2012). Adventitious agents, new technology, and risk assessment, 19–20 May 2011, Baltimore, MD. *Biologicals*, **40**, 162–167.

TELLEZ S., CASIMIRO R., VELA A.I., FERNANDEZ-GARAYZABAL J.F., EZQUERRA R., LATRE M.V., BRIONES V., GOYACHE J., BULLIDO R., ARBOIX M. & DOMINGUEZ L. (2005). Unexpected inefficiency of the European pharmacopoeia sterility test for detecting contamination in clostridial vaccines. *Vaccine,* **24**, 1710–1715.

VAN BORM S., ROSSEEL T., STEENSELS M., VAN DEN BERG T. & LAMBRECHT B. (2013). What's in a strain? Viral metagenomics identifies genetic variation and contaminating circoviruses in laboratory isolates of pigeon paramyxovirus type 1. *Virus Res,* **171**, 186–193.

VICTORIA J.G., WANG C., JONES M.S., JAING C., MCLOUGHLIN K., GARDNER S. & DELWART E.L. (2010). Viral nucleic acids in live-attenuated vaccines: detection of minority variants and an adventitious virus. *J. Virol.,* **84**, 6033–6040.

WANG J., LUNT R., MEEHAN B. & COLLING A. (2014). Evaluation of the potential role of next-generation sequencing (NGS) in innocuity testing. Technical Report CSIRO, Australian Animal Health Laboratory.

WORLD HEALTH ORGANIZATION (WHO) (1998). WHO Expert Committee on Biological Standardization. World Health Organization Technical Report Series, Report No. 858. World Health Organization, Geneva, Switzerland.

WORLD HEALTH ORGANIZATION (WHO) (2012). WHO Expert Committee on Biological Standardization. World Health Organization Technical Report Series, Report No. 964. World Health Organization, Geneva, Switzerland. Available online at: https://apps.who.int/iris/bitstream/handle/10665/89142/9789241209779_eng.pdf (Accessed 25 July 2023).

World Health Organisation (WHO) (2015) Expert Committee on Biological Standardization Sixty-fifth report. Technical report series 993, 2015. Annex 2 Scientific principles for regulatory risk evaluation on finding an adventitious agent in a marketed vaccine. Available online at: https://cdn.who.int/media/docs/default-source/biologicals/cell-

substrates/annex2__adventious_agent_in_marketed_vaccine_eng.pdf?sfvrsn=e5de04a_3&download=true (Accessed25 July 2023)

FURTHER READING

Details of methods and culture media ~~will~~ can be found in the following books and also in commercial catalogues.

BARROW G.I. & Feltham R.K.A., eds. (1993). Cowan and Steel's Manual for the Identification of Medical Bacteria, Third Edition. Cambridge University Press, Cambridge, UK.

COLLINS C.H., LYNE P.M. & GRANGE J.M., eds. (1995). Collins and Lyne's Microbiological Methods, Seventh Edition. Butterworth Heinemann, Oxford, UK.

MURRAY P.R., ED. (2003). Manual of Clinical Microbiology, Eighth Edition. American Society for Microbiology Press, Washington DC, USA.

*

* *

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

Annex 6. Chapter 2.2.4. 'Measurement uncertainty'

CHAPTER 2.2.4.

MEASUREMENT UNCERTAINTY

INTRODUCTION

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

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

A. THE NECESSITY OF DETERMINING MU

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

1. Samples for use in determining MU

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

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

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

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

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

2. Example of MU calculations in ELISA serology

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

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

2.1. Method of expression of MU

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

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

X represents the set of replicates

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

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

The relative standard deviation becomes:

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

2.2. Example

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

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

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

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

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

2.3. Calculating uncertainty

From the limited data set,

RSD (PI~~LW~~) = SD/Mean = 7.9/56.3 = 0.14 (or as coefficient of variation = 14%)

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

U (95% ~~C~~ RI) = 2 × RSD = 0.28

This estimate can then be applied at the threshold level

95% ~~C~~ RI = 50 ± (50 × 0.28) = 50 ± 14%

2.4. Interpretation of the results

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

3. Example of MU calculation in molecular tests

3.1. Example

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

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

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

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

3.2. Calculating uncertainty

From the limited data set,

RSD (PI_{LW}) = SD/Mean 0.43/33.36 = 0.0128 (or as coefficient of variation = 1.28%)

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

U (95% ~~C~~ RI) = 2 × RSD = 0.0255

This estimate can then be applied at the threshold level

95% ~~C~~ RI = 37± (37 × 0.0255) = 37 ± 0.94

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

3.3. Interpretation of the results

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

B. OTHER APPLICATIONS

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

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

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

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

Scope and limitations of the top-down approach

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

REFERENCES

~~American Association for Laboratory Accreditation (A2LA). Policy on estimating measurement uncertainty for life science testing labs https://portal.a2la.org/policies/A2LA_P103b.pdf (accessed 22 November 2018).~~

~~Australian Government, Department of Agriculture and Water Resources.. Worked examples of measurement uncertainty http://www.agriculture.gov.au/animal/health/laboratories/tests/worked-example-measurement (accessed 22 November 2018).~~

~~Australian Government Department of Health and Ageing (2007). Requirements for the estimation of measurement uncertainty, National Pathology Accreditation Advisory Group. http://www.health.gov.au/internet/main/publishing.nsf/Content/B1074B732F32282DCA257BF0001FA218/$File/dhaeou.pdf (accessed 22 November 2018).~~

Dimech W., Francis B., Kox J. & Roberts G. (2006). Calculating uncertainty of measurement for serology assays by use of precision and bias. *Clin. Chem*., **52**, No. 3, 526–529.

Ellison S.L.R. & Williams A. (eds) (2012). Eurachem/CITAC Guide CG 4: Quantifying uncertainty in analytical measurement, 3rd Edition. Eurachem, Teddington, United Kingdom, Section 3. Available at: www.eurachem.org/images/stories/Guides/pdf/QUAM2012_P1.pdf (Accessed 10 June 2023).

Goris N., Vandenbussche F., Herr, C., Villers, J., Van der Stede, Y. & De Clercq K. (2009). Validation of two real-time PCR methods for foot-and-mouth disease diagnosis: RNA-extraction, matrix effects, uncertainty of measurement and precision. *J. Virol. Methods,* **160**, 157–162.

Greiner M., Sohr D. & Goebel P.A. (1995). Modified ROC analysis for the selection of cut-off values and the definition of intermediate results of serodiagnostic tests, *J. Immunol. Methods*, **185**, 123–132.

ISO/IEC ~~(1995). Guide to the expression of uncertainty in measurement (GUM), ISO/IEC Guide 98:1995. International Organization for Standardization (ISO), www.iso.org.~~

~~ISO/IEC (2005). ISO/IEC~~ 17025:~~2005~~2017. General Requirements for the Competence of Testing and Calibration Laboratories. International Organization for Standardization (ISO), www.iso.org.

ISO/IEC Guide 98-3:2008 (E). Uncertainty of measurement – Part 3 Guide to the expression of uncertainty in measurement (GUM:1995), International Organization for Standardization (ISO), www.iso.org (accessed 10 June 2023).

SCC- Standards Council of Canada. Requirements and Guidance for method Verification and Validation in Testing Laboratories (RG-MVVT), 2021-05-24. http://www.scc.ca/ (accessed 10 June 2023) .

TOUSSAINT J.F., ASSAM P., CAIJ B., DEKEYSER F., KNAPEN K., IMBERECHTS H., GORIS N., MOLENBERGHS G., MINTIENS K., & DE CLERCQ K. (2007). Uncertainty of measurement for competitive and indirect ELISAs. *Rev. sci. tech. Off. int. Epiz.*, **26**, 649–656.

NEWBERRY K. & COLLING A (2021). Quality standards and guidelines for test validation for infectious diseases in veterinary laboratories. *Rev. Sci. Tech. Off. Int. Epiz.*, 40, 227–237. Available at https://doc.woah.org/dyn/portal/index.xhtml?page=alo&aloId=41245 (accessed 22 June 2023)

*
* *

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

NB: FIRST ADOPTED IN 2014.

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

CHAPTER 2.2.6.

SELECTION AND USE OF REFERENCE SAMPLES AND PANELS

INTRODUCTION

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

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

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

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

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

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

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

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

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

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

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

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

<table>
<tr><th>Pathogen data</th><th>Animal host and sample type data</th><th>Phase of Infection data</th></tr>
<tr><td rowspan="2">• Strain/isolate
• Serotype
• Genotype
• Lineage
• Tests used for characterisation</td><td>• Natural infection
• Experimental infection and protocol used
• Species
• Breed
• Age
• Sex
• Reproductive status
• Vaccination history
• Herd history</td><td rowspan="2">• Clinical signs
• infection/disease outcome
• Antibody profiles
• Pathogen loading and shedding
• Tests used to determine status of disease/infection (case definition)
• Time post-experimental infection</td></tr>
<tr><td>• Tissue type/s (matrix) used
• For spiked samples – detail source of analyte and diluent (matrix) used
• Details relating to pooling of samples</td></tr>
</table>

A. GROUP A

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

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

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

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

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

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

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

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

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

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

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

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

2.2. Comparative approaches to analytical sensitivity

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

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

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

3. Optimisation (~~WOAH Validation Standard,~~ Chapter 1.1.6, Section A.2.3~~2~~) and preliminary repeatability (~~WOAH Validation Standard,~~ Chapter 1.1.6, Section A.2.6~~8~~)

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

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

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

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

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

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

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

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

4.2. Working standards or process controls

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

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

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

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

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

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

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

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

B. GROUP B

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

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

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

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

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

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

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

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

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

C. GROUP C

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

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

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

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

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

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

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

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

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

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

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

D. GROUP D

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

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

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

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

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

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

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

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

E. GROUP E

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

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

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

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

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

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

F. GROUP F

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

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

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

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

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

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

FURTHER READING

BOWDEN T.R., CROWTHER J.R. & WANG J. (2021). Review of critical factors affecting analytical characteristics of serological and molecular assays. *Rev. Sci. Tech. Off. Int. Epiz.*, 40, 53–73. doi:10.20506/rst.40.1.3208.

CHEUNG A., DUFOUR S., JONES G., KOSTOULAS P., STEVENSON M.A., SINGANALLUR N.B. & FIRESTONE S.M. (2021). Bayesian latent class analysis when the reference test is imperfect. *Rev. Sci. Tech. Off. Int. Epiz.*, **40**, 271–286. doi:10.20506/rst.40.1.3224

JOHNSON P. & CABUANG L. (2021). Proficiency testing and ring trials. *Rev. Sci. Tech. Off. Int. Epiz.*, **40**, 189–203. https://doi.org/10.20506/rst.40.1.3217

HEUER C. & STEVENSON M.A. (2021). Diagnostic test validation studies when there is a perfect reference standard. *Rev. Sci. Tech. Off. Int. Epiz.*, **40**, 261–270. doi:10.20506/rst.40.1.3223

REISING M.M., TONG C., HARRIS B., TOOHEY-KURTH K.L., CROSSLEY B., MULROONEY D., TALLMADGE R.L., SCHUMANN K.R., LOCK, A.B. & LOIACONO C.M. (2021). A review of guidelines for evaluating a minor modification to a validated assay. *Rev. Sci. Tech. Off. Int. Epiz.*, **40**, 217–226. doi:10.20506/rst.40.1.3219

WATSON J.W., CLARK G.A. & WILLIAMS D.T. (2021). The value of virtual biobanks for transparency purposes with respect to reagents and samples used during test development and validation. *Rev. Sci. Tech. Off. Int. Epiz.*, **40**, 253–259. doi:10.20506/rst.40.1.3222.

WAUGH C. & CLARK G. (2021). Factors affecting test reproducibility among laboratories. *Rev. Sci. Tech. Off. Int. Epiz.*, **40**, 131–143. doi:10.20506/rst.40.1.3213

*
* *

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

NB: FIRST ADOPTED IN 2014.

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

CHAPTER 3.1.5.

CRIMEAN–CONGO HAEMORRHAGIC FEVER

SUMMARY

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

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

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

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

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

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

A. INTRODUCTION

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

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

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

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

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

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

B. DIAGNOSTIC TECHNIQUES

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

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

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

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

1. Detection and identification of the agent

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

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

1.1. Virus isolation in cell culture

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

1.1.1. Test procedure

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

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

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

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

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

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

1.2. Nucleic acid detection

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

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

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

Clade	Molecular assay combinations	Primer and probe names (5' → 3' sequence)
Africa 1	Real-time RT-PCR	Fwd CCRealP1 (TCT-TYG-CHG-ATG-AYT-CHT-TYC) Rev CCRealP2 (GGG-ATK-GTY-CCR-AAG-CA) Probe (ACA-SRA-TCT-AYA-TGC-AYC-CTG-C)
Africa 2	Real-time RT-PCR	Fwd CCRealP1 (TCT-TYG-CHG-ATG-AYT-CHT-TYC) Rev CCRealP2 (GGG-ATK-GTY-CCR-AAG-CA) Probe (ACA-SRA-TCT-AYA-TGC-AYC-CTG-C)
	Real-time RT-PCR	Fwd CCHF-SF2 (GGA-VTG-GTG-VAG-GGA-RTT-TG) Rev CCHF-SR2 (CAD-GGT-GGR-TTG-AAR-GC) Fwd CCHF-N2 (CAA-RGG-CAA-RTA-CAT-MAT)
Africa 3	Nested RT-PCR	Fwd CCHF1 (CTG-CTC-TGG-TGG-AGG-CAA-CAA) Rev CCHF2_5 (TGG-GTT-GAA-GGC-CAT-GAT-GTA-T) Nested Fwd CCHFn15 (AGG-TTT-CCG-TGT-CAA-TGC-AAA) Nested Rev CCHFn25 (TTG-ACA-AAC-TCC-CTG-CAC-CAG-T)
	Nested RT-PCR	Fwd CrCon1+ (RWA-AYG-GRC-TTR-TGG-AYA-CYT-TCA-C) Rev CrCon1– (TRG-CAA-GRC-CKG-TWG-CRA-CWA-GWG-C) Nested Fwd CriCon2+ (ART-GGA-GRA-ARG-AYA-TWG-GYT-TYC-G) Nested Rev CriCon2– (CYT-TGA-YRA-AYT-CYC-TRC-ACC-ABT-C)
Africa 4	Real-time RT-PCR	Fwd CCRealP1 (TCT-TYG-CHG-ATG-AYT-CHT-TYC) Rev CCRealP2 (GGG-ATK-GTY-CCR-AAG-CA) Probe (ACA-SRA-TCT-AYA-TGC-AYC-CTG-C)
	Real-time RT-PCR	Fwd CCHF-III (CAA-GAG-GTA-CCA-AGA-AAA-TGA-AGA-AGG-C) Rev CCHF-III-r (GCC-ACG-GGG-ATT-GTC-CCA-AAG-CAG-AC) Probe CCHFprobe-1 (ATC-TAC-ATG-CAC-CCT-GCY-GTG-YTG-ACA) Probe CCHFprobe-2 (TTC-TTC-CCC-CAC-TTC-ATT-GGR-GTG-CTC-A)
Asia 1	Nested PCR	Fwd CCF-115F (AAR-GGA-AAT-GGA-CTT-RTG-GA) Fwd CCF-131F (TGG-AYA-CYT-TCA-CAA-ACT-CC) Rev CCF-759R (GCA-AGG-CCT-GTW-GCR-ACA-AGT-GC)
	Real-time RT-PCR	Fwd CC1a_for (GTG-CCA-CTG-ATG-ATG-CAC-AAA-AGG-ATT-CCA-TCT) Rev CC1a_rev (GTG-CCA-CTG-ATG-ATG-CAC-AAA-AGG-ATT-CCA-TCT) Probe CCHF-01 (CAA-CAG-GCT-GCT-CTC-AAG-TGG-AG)
	Real-time RT-PCR	Fwd CCHF-SF2 (GGA-VTG-GTG-VAG-GGA-RTT-TG) Rev CCHF-SR2 (CAD-GGT-GGR-TTG-AAR-GC) Probe CCHF-N2 (CAA-RGG-CAA-RTA-CAT-MAT)
Asia 2	Nested PCR	Fwd CCF-115F (AAR-GGA-AAT-GGA-CTT-RTG-GA) Fwd CCF-131F (TGG-AYA-CYT-TCA-CAA-ACT-CC) Rev CCF-759R (GCA-AGG-CCT-GTW-GCR-ACA-AGT-GC)
	Sybrgreen Real-time RT-PCR	Fwd (GAT-GAG-ATG-AAC-AAG-TGG-TTT-GAA-GA) Rev (GTA-GAT-GGA-ATC-CTT-TTG-TGC-ATC-AT)
	RT-PCR	Fwd CCS (ATG-CAG-GAA-CCA-TTA-ART-CTT-GGG-A) Rev 1 CCAS1 (CTA-ATC-ATA-TCT-GAC-AAC-ATT-TC) Rev 2 CCAS2 (CTA-ATC-ATG-TCT-GAC-AGC-ATC-TC)
Europe 1	Real-time RT-PCR	Fwd CCRealP1 (TCT-TYG-CHG-ATG-AYT-CHT-TYC) Rev CCRealP2 (GGG-ATK-GTY-CCR-AAG-CA) Probe (ACA-SRA-TCT-AYA-TGC-AYC-CTG-C)
	Nested RT-PCR	Fwd CCF-115F (AAR-GGA-AAT-GGA-CTT-RTG-GA)

Clade	Molecular assay combinations	Primer and probe names (5' → 3' sequence)
		Fwd CCF-131F (TGG-AYA-CYT-TCA-CAA-ACT-CC) Rev CCF-759R (GCA-AGG-CCT-GTW-GCR-ACA-AGT-GC)
Europe 2	Nested RT-PCR	Fwd CrCon1+ (RWA-AYG-GRC-TTR-TGG-AYA-CYT-TCA-C) Rev CrCon1– (TRG-CAA-GRC-CKG-TWG-CRA-CWA-GWG-C) Fwd CriCon2+ (ART-GGA-GRA-ARG-AYA-TWG-GYT-TYC-G) Rev CriCon2– (CYT-TGA-YRA-AYT-CYC-TRC-ACC-ABT-C)
Europe 3	Real-time RT-PCR	Fwd CCRealP1 (TCT-TYG-CHG-ATG-AYT-CHT-TYC) Rev CCRealP2 (GGG-ATK-GTY-CCR-AAG-CA) Probe (ACA-SRA-TCT-AYA-TGC-AYC-CTG-C)
All	Real-time RT-PCR	Fwd CCRealP1 (TCT-TYG-CHG-ATG-AYT-CHT-TYC) Rev CCRealP2 (GGG-ATK-GTY-CCR-AAG-CA) Probe (ACA-SRA-TCT-AYA-TGC-AYC-CTG-C)
	Nested RT-PCR	Fwd CrCon1+ (RWA-AYG-GRC-TTR-TGG-AYA-CYT-TCA-C) Rev CrCon1– (TRG-CAA-GRC-CKG-TWG-CRA-CWA-GWG-C) Rev Fwd CriCon2+ (ART-GGA-GRA-ARG-AYA-TWG-GYT-TYC-G) Nested Rev CriCon2– (CYT-TGA-YRA-AYT-CYC-TRC-ACC-ABT-C)
	Real-time RT-PCR	Fwd CCHF-SF2 (GGA-VTG-GTG-VAG-GGA-RTT-TG) Rev CCHF-SR2 (CAD-GGT-GGR-TTG-AAR-GC) Probe CCHF-N2 (CAA-RGG-CAA-RTA-CAT-MAT)
	RT-PCR	Fwd CCS (ATG-CAG-GAA-CCA-TTA-ART-CTT-GGG-A) Rev 1 CCAS1 (CTA-ATC-ATA-TCT-GAC-AAC-ATT-TC) Rev 2 CCAS2 (CTA-ATC-ATG-TCT-GAC-AGC-ATC-TC)
	Real-time RT-PCR	Fwd CC1a_for (GTG-CCA-CTG-ATG-ATG-CAC-AAA-AGG-ATT-CCA-TCT) Rev CC1a_rev (GTG-CCA-CTG-ATG-ATG-CAC-AAA-AGG-ATT-CCA-TCT) Probe CCHF-01 (CAA-CAG-GCT-GCT-CTC-AAG-TGG-AG)

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

2. Serological tests

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

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

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

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

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

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

C. REQUIREMENTS FOR VACCINES

There is no vaccine available for animals.

REFERENCES

AVSIC-ZUPANC T. (2007). Epidemiology of Crimean–Congo hemorrhagic fever in the Balkans. *In:* Crimean–Congo Hemorrhagic Fever, a Global Perspective, Ergonul O. & Whitehouse C.A., eds. Springer: Dordrecht, Netherlands, 75–88.

BELL-SAKYI L., KOHL D., BENTE D.A. & FAZAKERLEY J.F. (2012). Tick cell lines for study of Crimean–Congo hemorrhagic fever virus and other arboviruses. Vector Borne Zoonotic Dis., **12**, 769–781.

BURT F.J., LEMAN P.A., ABBOTT J.C. & SWANEPOEL R. (1994). Serodiagnosis of Crimean-Congo haemorrhagic fever. *Epidemiol. Infect*., **113**, 551–562. Doi: 10.1017/s0950268800068576

BURT F.J., LEMAN P.A., SMITH J.F. & SWANEPOEL R. (1998). The use of a reverse transcription-polymerase chain reaction for the detection of viral nucleic acid in the diagnosis of Crimean–Congo haemorrhagic fever. *J. Virol. Methods*, **70**, 129–37.

DROSTEN C., GOTTING S., SCHILLING S., ASPER M., PANNING M., SCMITZ H. & GUNTER S. (2002). Rapid detection and quantification of RNA of Ebola and Marburg viruses, Lassa virus, Crimean–Congo hemorrhagic fever virus, Rift Valley fever virus, dengue virus, and yellow fever virus by real-time transcription-PCR. *J. Clin. Microbiol*., **40**, 2323–2340.

DROSTEN C., KUMMERER B.M., SCMITZ H. & GUNTER S. (2003). Molecular diagnosis of viral hemorrhagic fevers. *Antiviral Res.,* **57**, 61–87.

DUH D., SAKSIDA A., PETROVEC M., DEDUSHAJ I. & AVSIC-ZUPANC T. (2006). Novel one-step real-time RT-PCR assay for rapid and specific diagnosis of Crimean–Congo hemorrhagic fever encountered in the Balkans. *J. Virol. Methods*, **133**, 175–179.

EMMERICH P., MIKA A., VON POSSEL R., RACKOW A., LIU Y., SCHMITZ H., GÜNTHER S., SHERIFI K., HALILI B., JAKUPI X., BERISHA L., AHMETI S. & DESCHERMEIER C. (2018). Sensitive and specific detection of Crimean–Congo Hemorrhagic Fever Virus (CCHFV)-Specific IgM and IgG antibodies in human sera using recombinant CCHFV nucleoprotein as antigen in µ-capture and IgG immune complex (IC) ELISA tests. PLoS Negl. Trop. Dis., **12**(3):e0006366. doi: 10.1371/journal.pntd.0006366.

EMMERICH P., VON POSSEL R., DESCHERMEIER C., AHMETI S., BERISHA L., HALILI B., JAKUPI X., SHERIFI K., MESSING C. & BORCHARDT-LOHÖLTER V. (2021). Comparison of diagnostic performances of ten different immunoassays detecting anti-CCHFV IgM and IgG antibodies from acute to subsided phases of Crimean–Congo hemorrhagic fever. *PLoS Negl. Trop Dis*., **15** (3):e0009280.

ERGONUL O. (2006). Crimean–Congo haemorrhagic fever. *Lancet Infect. Dis.,* **6**, 203–214.

GONZALEZ J.-P., CAMICAS J.-L., COMET J.-P. & WILSON M.L. (1998). Biological and clinical responses of West African sheep to Crimean–Congo haemorrhagic fever virus experimental infection. *Res. Virol*., **149**, 445–455.

GUNES T., POYRAZ O., VATANSEVER Z. (2011). Crimean–Congo hemorrhagic fever virus in ticks collected from humans, livestock, and picnic sites in the hyperendemic region of Turkey. *Vector Borne Zoonotic Dis.*, **11**, 1411–1416.

GRARD G., DREXLER J.F., FAIR J., MUYEMBE J.-J., WOLFE N.D., DROSTEN C. & LEROY E.M. (2011). Re-emergence of
Crimean–Congo hemorrhagic fever virus in Central Africa. *PLoS Negl. Trop. Dis*., **5**(10): e1350. doi:10.1371/journal.pntd.0001350.

GRUBER C., BARTOLINI B., CASTILLETTI C., MIRAZIMI A., HEWSON R., CHRISTOVA I., AVŠIČ T., GRUNOW R., PAPA A., SÁNCHEZ-SECO M. P., KOPMANS M., IPPOLITO G., CAPOBIANCHI M. R., REUSKEN C. & DI CARO A. (2019). Geographical Variability Affects CCHFV Detection by RT-PCR: A Tool for *In-Silico* Evaluation of Molecular Assays. *Viruses*, **11**, 953.

GULCE-İZ S., ELALDI N., CAN H Şahar E.A., Karakavuk M., Gül A., Kumoğlu G.Ö., Döşkaya A.D., Gürüz A.Y., Özdarendeli A., Felgner P.L., Davies H. & Döşkaya M. (2021). Development of a novel recombinant ELISA for the detection of Crimean–Congo hemorrhagic fever virus IgG antibodies. *Sci. Rep*., **11**, 5936. doi:10.1038/s41598-021-85323-1.

HOOGSTRAAL H. (1979). The epidemiology of tick-borne Crimean–Congo haemorrhagic fever in Asia, Europe and Africa. *J. Med. Entomol*., **15**, 307–417.

KOEHLER J.W., DELP K.L., HALL A.T., OLSCHNER S.P., KEARNEY B.J., GARRISON A.R., ALTAMURA L.A., ROSSI C.A. & MINOGUE T.D. (2018). Sequence Optimized Real-Time Reverse Transcription Polymerase Chain Reaction Assay for Detection of Crimean–Congo Hemorrhagic Fever Virus. *Am. J. Trop. Med. Hyg.,* **98**, 211–215.

MERTENS M., SCHMIDT K., OZKUL A. & GROSCHUP M.H. (2013). The impact of Crimean–Congo hemorrhagic fever virus on public health. *Antiviral Res*., **98**, 248–260.

NALCA A. & WHITEHOUSE C.A. (2007). Crimean–Congo hemorrhagic fever virus infection among animals. *In:* Crimean–Congo Hemorrhagic Fever: A Global Perspective, Ergonul O. & Whitehouse C.A., eds. Springer: Dordrecht, Netherlands, 155–165.

NEGREDO A., DE LA CALLE-PRIETO F., PALENCIA-HERREJÓN E., MORA-RILLO M., ASTRAY-MOCHALES J., SÁNCHEZ-SECO M.P., BERMEJO LOPEZ E., MENÁRGUEZ J., FERNÁNDEZ-CRUZ A., SÁNCHEZ-ARTOLA B., KEOUGH-DELGADO E., RAMÍREZ DE ARELLANO E., LASALA F., MILLA J., FRAILE J.L., ORDOBÁS GAVÍN M., MARTINEZ DE LA GÁNDARA A., LÓPEZ PEREZ L., DIAZ-DIAZ D., LÓPEZ-GARCÍA M.A., DELGADO-JIMENEZ P., MARTÍN-QUIRÓS A., TRIGO E., FIGUEIRA J.C., MANZANARES J., RODRIGUEZ-BAENA E., GARCIA-COMAS L., RODRÍGUEZ-FRAGA O., GARCÍA-ARENZANA N., FERNÁNDEZ-DÍAZ M.V., CORNEJO V.M., EMMERICH P., SCHMIDT-CHANASIT J., ARRIBAS J.R., CRIMEAN CONGO HEMORRHAGIC FEVER@MADRID WORKING GROUP (2017). Autochthonous Crimean–Congo Hemorrhagic Fever in Spain. *N. Engl. J. Med*., **377**, 154–161. 10.1056/NEJMoa1615162.

PALMER S. (2011). Deliberate release of zoonotic agents. *In:* Oxford Textbook of Zoonosis: Biology, Clinical Practise and Public Health Control, Second Edition, Palmer S.R., Soulsby L., Torgerson P.R. & Brown D.W.G., eds. Oxford University Press, UK, p. 1214.

PAPA A., TZALA E. & MALTEZOU H. (2011). Crimean–Congo hemorrhagic fever virus, Northeastern Greece. *Emerg. Infect. Dis*., **17**, 141–143.

RODRIGUEZ L.L., MAUPIN G.O., KSIAZEK T.G., ROLLIN P.E., KHAN A.S., SCHWARZ T.F., LOFTS R.S., SMITH J.F., NOOR A.M., PETERS C.J. & NICHOL S.T. (1997). Molecular investigation of a multisource outbreak of Crimean–Congo hemorrhagic fever in the United Arab Emirates. *Am. J. Trop. Med. Hyg*., 57, 512–518.

SAS M.A., VINA-RODRIGUEZ A., MERTENS M., EIDEN M., EMMERICH P., CHAINTOUTIS S.C., MIRAZIMI A. & GROSCHUP M.H. (2018). A one-step multiplex real-time RT-PCR for the universal detection of all currently known CCHFV genotypes. *J. Virol. Methods*, **255**, 38–43.

SHEPHERD A.J., SWANEPOEL R. & GILL D.E. (1988). Evaluation of enzyme-linked immunosorbent assay and reversed passive hemagglutination for detection of Crimean–Congo hemorrhagic fever virus antigen. *J. Clin. Microbiol.*, **26**, 347–353.

SHEPHERD A.J., SWANEPOEL R., LEMAN P.A. & SHEPHERD S.P. (1986). Comparison of methods for isolation and titration of Crimean-Congo hemorrhagic fever virus. *J. Clin. Microbiol.*, **24**, 654–656.

SPENGLER J.R., ESTRADA-PEÑA A., GARRISON A.R., SCHMALJOHN C., SPIROPOULOU C.F., BERGERON É. & BENTE D.A. (2016). A chronological review of experimental infection studies of the role of wild animals and livestock in the maintenance and transmission of Crimean-Congo hemorrhagic fever virus. *Antiviral Res.*, **135**, 31–47. doi: 10.1016/j.antiviral.2016.09.013.

SWANEPOEL R & BURT F.J. (2004). Crimean–Congo haemorrhagic fever. Second Edition. *In:* Infectious diseases of livestock with special reference to South Africa, Coetzer J.A.W, Tustin R.C., eds. Cape Town: Oxford University Press Southern Africa, pp. 1077–1085.

SWANEPOEL R., LEMAN P.A., BURT, F.J., JARDINE J., VERWOERD D.J., CAPUA I., BRUCKNER G.K. & BURGER W.P. (1998). Experimental infection of ostriches with Crimean-Congo haemorrhagic fever virus. *Epidemiol. Infect.*, **121**, 427–432.

SWANEPOEL R. & PAWESKA J.T. (2011). Crimean-Congo hemorrhagic fever. *In:* Oxford Textbook of Zoonosis: Biology, Clinical Practise and Public Health Control, Second Edition. Palmer S.R., Soulsby L., Torgerson P.R. & Brown D.W.G.,eds. Oxford University Press, UK, pp. 287–293.

WHITEHOUSE C.A. (2004). Crimean–Congo hemorrhagic fever. *Antivir. Res.*, **64**, 145–160.

WOLFEL R., PAWESKA J.T., PETERSEN N., GROBBELAAR A.G., LEMAN P.A., HEWSON R., GEORGES-COURBOT, M., PAPA, A., GÜNTER S. & DROSTEN C. (2007). Virus detection and monitoring of viral load in Crimean-Congo hemorrhagic fever virus patients. *Emerg. Infect. Dis.,* **13**, 1097–1100.

WOLFEL R., PAWESKA J.T., PETERSEN N., GROBBELAAR A.G., LEMAN P.A., HEWSON R., GEORGES-COURBOT M., PAPA, A., HEISER V., PANNING M., GUNTER S. & DROSTEN C. (2009). Low-density microarray for rapid detection and identification of Crimean–Congo hemorrhagic fever virus. *J. Clin. Microbiol.*, **47**, 1025–1030.

YEN Y.C., KONG L.X., LEE L., ZHANG Y.Q., LI F., CAI B.J. & GAO S.Y. (1985). Characteristics of Crimean–Congo hemorrhagic fever virus (Xinjiang strain) in China. *Am. J. Trop. Med. Hyg.*, **34**, 1179**–**1182.

YILMAZ G.R., BUZGAN T., TORUNOGLU M.A., SAFRAN A., IRMAK H., COM S., UYAR Y., CARHAN A., OZKAYA E. & ERTEK M. (2008). A preliminary report on Crimean**–**Congo haemorrhagic fever in Turkey, March**–**June 2008. *Euro Surveill.*, 13.

*
* *

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

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

Annex 9. Chapter 3.3.6. 'Avian tuberculosis'

CHAPTER 3.3.6.

AVIAN TUBERCULOSIS

SUMMARY

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

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

Mycobacterium tuberculosis*, the agent that most commonly causes human tuberculosis* (*gene IS*6110*1*) *is ~~less commonly~~ rarely the cause of infection in birds, and it is often ~~as a~~ the result of transmission from pet bird owners* or caretakers of captive birds.

Members of M. avium *complex:* M. a. avium (*serotypes 1–3; containing ~~gene segments~~ insertion sequences IS*901 *and IS*1245), M. avium *subsp.* hominissuis (*serotypes 4–6, 8–11, and 21; lacking gene segment IS*901 *and containing segment IS*1245) *and* M. intracellulare (*serotypes 7, 12–20, and 22–28; lacking both IS*901 *and IS*1245) *can also infect an extensive range of mammals such as swine, cattle, deer, sheep, goats, horses, cats, dogs, and exotic species. In humans, all members of the* M. avium *complex and* M. genavense *~~are capable of inducing~~ can induce a progressive disease that is refractory to treatment, ~~mostly~~ mainly in immunocompromised patients.*

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

Diagnosis of avian tuberculosis in birds depends on the demonstration of ~~the above-mentioned~~ a mycobacterial species *in live or dead birds or the detection of an immune response, cellular or humoral, culture examination, or gene segments ~~IS~~6110*, ~~IS~~901 and ~~IS~~1245 by polymerase chain reaction (PCR) in the excretions or secretions of live birds.*

Detection of the agent: *Where clinical signs of avian tuberculosis are seen in the flock, or typical tuberculous lesions are present in birds at necropsy, the demonstration of acid-fast bacilli in smears or sections made from affected organs is sufficient for a quick positive diagnosis. If acid-fast bacilli are not found but typical tuberculous signs or lesions are present in the birds, a culture of the organism or PCR must be attempted~~. PCR could also be carried out directly~~ on tissue samples. Any acid-fast organism isolated should be identified by nucleic-acid-based tests or chromatographical (e.g. high-performance liquid chromatography [HPLC]) criteria. Serotyping of isolates of* M. avium *complex members or PCR for 16S rRNA gene followed by sequencing, or the presence of an amplicon for the insertion sequences IS*6110*, IS*901, *and IS*1245 *~~could~~ can also be performed. Matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS) is a valuable tool as well for isolates following culture.*

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

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

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

A. INTRODUCTION

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

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

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

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

~~worth noting that the typical features of bird-isolates, the three-band pattern in IS*1245* RFLP and presence of IS*901*, have also been found in cervine and bovine isolates of *M. a. avium*.~~

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

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

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

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

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

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

immunosuppressed. All laboratory manipulations with live cultures or potentially infected/contaminated material must be performed at an appropriate biosafety and containment level determined by conducting a thorough risk assessment as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*. ~~Biocontainment measures should be determined by risk analysis as described in Chapter 1.1.4.~~ The CDC's online Manual for Biosafety in Microbiological and Biomedical Laboratories is also a good reference[2].

B. DIAGNOSTIC TECHNIQUES

Table 1. *Test methods available for the diagnosis of avian tuberculosis and their purpose*

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection and identification of the agent[(a)]						
Ziehl–Neelsen staining	–	–	–	++	–	–
Culture	–	–	–	++	–	–
~~Haemagglutination (stained antigen)~~	~~+~~	~~+++~~	~~+~~	–	~~++~~	–
PCR	++~~+~~	~~–~~ +	~~++~~ –	+++	~~+~~ –	–
Detection of immune response						
Haemagglutination (stained antigen)	+	+++	+	=	++	=
Tuberculin test	++	+++	+	–	++	–

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

1. Identification of the agent

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

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

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

1.1. Culture

If there is a characteristic flock history and suggestive lesions are found at necropsy, but no AFB are seen in smears or sections, an attempt must be made to isolate the causative organism from the necropsy material. The liver or spleen is usually the best organ to use, but if the carcass is decomposed, bone marrow may prove more satisfactory as it could be less contaminated. As with the culture of *M. bovis*, non-sterile specimens need to be processed with detergent, alkali, or acid to eliminate rapidly growing microorganisms before culture (see Chapter 3.1.13 *Mammalian tuberculosis* [*infection with* Mycobacterium tuberculosis *complex*]). *Mycobacterium a. avium* grows best on media such as Lowenstein–Jensen, Herrold's medium, Middlebrook 7H10 ~~and~~, 7H11, or Coletsos, with 1% sodium pyruvate added. It ~~may occasionally be~~ is necessary to incorporate mycobactin J, as it is used ~~for the isolation of~~ to isolate *M. ~~a. paratuberculosis~~ genavense* and *M. a. silvaticum.* ~~Growth may be confined to the edge of the condensation water.~~ Cultures should be incubated for at least ~~8~~ 12 weeks, less if using liquid media. Typically, *M. a. avium* produces 'smooth' colonies within 2–4 weeks; rough variants ~~do~~ occur. Shorter incubation times can be achieved using the liquid culture ~~BACTEC~~ system or the automated fluorescent ~~MGIT 960~~ culture system. *Mycobacterium a. avium* can also be detected in ~~massively~~ infected tissue by a conventional PCR, which also ~~allows acceleration of the~~ accelerates pathogen detection and identification (Moravkova *et al.,* 2008). ~~Currently,~~ Direct detection and quantification of *M. a. avium* using IS*901* quantitative real-time PCR can be considered ~~as~~ the ~~best~~ fast and inexpensive method ~~(despite its rather high cost per test)~~ (Kaevska *et al.,* 2010; Slana *et al.,* 2010).

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

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

1.2. Nucleic acid recognition methods

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

method for ~~distinction between~~ distinguishing *M. avium*, ~~and~~ *M. intracellulare* ~~cultures.~~ and *M. genavense* ~~can also be distinguished with these tests~~. A further probe that covers the whole *M. avium* complex was also developed, as genuine *M. avium* complex strains have been described that fail to react with specific *M. avium* and *M. intracellular* probes (Soini *et al*., 1996). Nevertheless, identification errors were reported due to the cross-reactivity, which may have serious consequences (van Ingen *et al.,* 2009). Various in-house molecular methods have been reported ~~for the identification of~~ to identify mycobacterial cultures, including ~~MAC.~~ members of the *Mycobacterium avium* complex. The following gene segments could be used to identify *Mycobacterium* isolates as *M. avium* in one multiplex PCR reaction: IS*900*, IS*901*, IS*1245*. The isolates of *M. a. avium/M. a. silvaticum* are IS*900*−, IS*901*+, IS*1245*+, the isolates of *M. a. hominissuis* are IS*900*−, IS*901*−, IS1*245*+, and the isolates of *M. a. paratuberculosis* are IS*900*+, IS*901*−, IS*1245*− (Kaevska *et al.*, 2010; Moravkova *et al.*, 2008). A ~~multiplex~~ 16S rRNA PCR and sequencing method for differentiating *M. avium* from *M. intracellulare* and *M. tuberculosis* complex ~~has some advantages (Cousins *et al*., 1996). 16S rRNA~~ is currently commercially available. Similarly, many veterinary diagnostic laboratories commonly perform in-house PCR and sequencing (Kirschner *et al*., 1993) ~~may also be used~~. Culture-independent in-house molecular tests have been developed ~~for the detection~~ to detect and ~~identification of~~ identify species belonging to the *M. avium* complex directly from samples (Hall *et al.*, 2003; Kaevska *et al.,* 2010). WGS of isolates has recently become the go-to molecular method to identify mycobacterium isolates from birds with great accuracy. It enables, with the use of bioinformatic tools, not only an accurate identification of species and subspecies, but also helps to determine the organism relatedness within a flock or environment (Witte *et al.,* 2021). In recent years, veterinary diagnostic laboratories have extensively adopted real-time PCR methods to detect *M. a. avium* directly from different specimens (faeces, tissues, formalin-fixed tissues, and environmental samples). The technique rapidly detects fastidious and slow-growing microorganisms, such as *M. a. avium* (Tell *et al.,* 2003a; 2003b).

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

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

2. Immunological methods

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

2.1. Tuberculin test

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

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

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

2.2. Stained antigen test

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

2.2.1. Preparation of the antigen

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

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

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

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

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

original method in which the washing was carried out before the incubation that kills the organisms. Finally, bacilli are again centrifuged and re-suspended in sterile normal saline containing 0.2% formalin and 0.4% sodium citrate to a concentration of about 10^{10} bacteria per ml. This corresponds to a concentration ten times that which matches tube No. 4 on McFarland's scale.

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

2.2.2. Validation of the antigen

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

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

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

Note on limitation of use

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

C. REQUIREMENTS FOR DIAGNOSTIC BIOLOGICALS

1. Background

No vaccines are available.

Avian tuberculin is a preparation of purified protein derivatives (PPD-A) made from the heat-treated products of growth of *M. a. avium*. It is used by intradermal injection to reveal delayed hypersensitivity ~~as a means of identifying~~ to identify birds infected with or sensitised to the same species of ~~tubercle bacillus~~ *Mycobacterium*. Importantly it is also used ~~as an~~ to aid ~~to~~ differential diagnosis in the comparative intradermal tuberculin test for bovine tuberculosis (see Chapter 3.1.13). An international standard preparation of PPD-A is being developed by WOAH to replace the former WHO Standard[3].

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

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

2. Outline of production and minimum requirements for tuberculin production

2.1. Characteristics of the seed

2.1.1. Biological characteristics of the master seed

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

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

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

2.2. Method of manufacture

2.2.1. Procedure

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

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

2.2.2. Requirements for ingredients

The production culture substrate must be shown to ~~be capable of producing~~ produce a product that conforms to the ~~standards of the~~ European Pharmacopoeia (~~2000~~ 2024[4]) standards or other international standards such the WHO (WHO, 1987). It must be free from ingredients known to cause toxic or allergic reactions.

2.2.3. In-process controls

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

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

2.2.4. Final product batch tests

i) Sterility

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

ii) Identity

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

iii) Safety

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

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

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

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

iv) Batch potency

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

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

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

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

3. Requirements for authorisation/registration/licensing

3.1. Manufacturing process

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

3.2. Safety requirements

3.2.1. Target and non-target animal safety

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

3.2.2. Precautions (hazards)

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

3.3. Stability

During storage, liquid avian tuberculin should be protected from the light and held at a temperature of 5°C (±3°C). Freeze-dried preparations may be stored at higher temperatures (~~but~~ not exceeding 25°C) and protected from ~~the~~ light. During use, periods of exposure to higher temperatures or to direct sunlight should be kept at a minimum.

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

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

REFERENCES

ANGUS R.D. (1978). Production of Reference PPD tuberculins for Veterinary use in the United States. *J. Biol. Stand.*, **6**, 221.

ARAHAL D.R., BULL C.T., BUSSE H.J., CHRISTENSEN H., CHUVOCHINA M., DEDYSH S.N., FOURNIER P.E., KONSTANTINIDIS K.T., PARKER C.T., ROSSELLO-MORA R., VENTOSA A. & GOKER M. (2023). Guidelines for interpreting the International Code of Nomenclature of Prokaryotes and for preparing a Request for an Opinion. *Int. J. Syst. Evol. Microbiol.*, **73**.

BUCKWALTER S.P., OLSON S.L., CONNELLY B.J., LUCAS B.C., RODNING A.A., WALCHAK R.C., DEML S.M., WOHLFIEL S.L. & WENGENACK N.L. (2016). Evaluation of Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry for Identification of *Mycobacterium* species, *Nocardia* species, and Other Aerobic *Actinomycetes*. *J. Clin. Microbiol.,* **54**, 376–384.

~~COUSINS D., FRANCIS B. & DAWSON D. (1996). Multiplex PCR provides a low-cost alternative to DNA probe methods for rapid identification of *Mycobacterium avium* and *Mycobacterium intracellulare*. *J. Clin. Microbiol.,* **34**, 2331–2333.~~

COWMAN S., VAN INGEN J., GRIFFITH D.E. & LOEBINGER M.R. (2019). Non-tuberculous mycobacterial pulmonary disease. *Eur .Respir. J.,* **54**(1).

~~DVORSKA L., BULL T.J., BARTOS M., MATLOVA L., SVASTOVA P., WESTON R.T., KINTR J., PARMOVA I., VAN SOOLINGEN D. & PAVLIK I. (2003). A standardised restriction fragment length polymorphism (RFLP) method for typing *Mycobacterium avium* isolates links IS*901* with virulence for birds. *J. Microbiol. Methods,* **55**, 11–27.~~

DVORSKA L., MATLOVA L., AYELE W. Y., FISCHER O. A., AMEMORI T., WESTON R. T., ALVAREZ J., BERAN V., MORAVKOVA M. & PAVLIK I. (2007). Avian tuberculosis in naturally infected captive water birds of the Ardeidae and Threskiornithidae families studied by serotyping, IS*901* RFLP typing and virulence for poultry. *Vet. Microbiol.*, **119**, 366–374.

DVORSKA L., MATLOVA L., BARTOS M., PARMOVA I., BARTL J., SVASTOVA P., BULL T. J. & PAVLIK I. (2004). Study of *Mycobacterium avium* complex strains isolated from cattle in the Czech Republic between 1996 and 2000. *Vet. Microbiol.,* **99**, 239–250.

~~DVORSKA L., PARMOVA I., LAVICKOVA M., BARTL J., VRBAS V. & PAVLIK I. (1999). Isolation of *Rhodococcus equi* and atypical mycobacteria from lymph nodes of pigs and cattle in herds with the occurrence of tuberculoid gross changes in the Czech Republic over the period of 1996-1998. *Veterinarni Medicina,* **44**, 321–330.~~

EUROPEAN PHARMACOPOEIA (~~2000~~ 2024). Purified protein derivative (avian). *In:* European Pharmacopoeia, Eleventh Edition. Editions of the Council of Europe, Strasbourg, France, https://www.edqm.eu/en/d/234640?p_l_back_url=%2Fen%2Fsearch%3Fq%3Dpurified%2Bprotein%2Bderivative

FERNÁNDEZ-ESGUEVA M., FERNÁNDEZ-SIMON R., MONFORTE-CIRAC M.L., LÓPEZ-CALLEJA A.I., FORTUNO B. & VINUELAS-BAYON J. (2021). Use of MALDI-TOF MS (Bruker Daltonics) for identification of *Mycobacterium* species isolated directly from liquid medium. *Enferm. Infecc. Microbiol. Clin.* (*Engl. Ed.*) **39**, 241–243. doi: 10.1016/j.eimc.2020.05.011.

GUERRERO C., BERNASCONI C., BURKI D., BODMER T. & TELENTI A. (1995). A novel insertion element from *Mycobacterium avium,* IS*1245*, is a specific target for analysis of strain relatedness. *J. Clin. Microbiol.,* **33**, 304–307.

HAAGSMA J. & ANGUS R.D. (1995). Tuberculin production. *In: Mycobacterium bovis* Infections in Humans and Animals, Steele J.H. & Thoen C.O., eds. Iowa State University Press, Ames, USA, 73–84.

HALL L., DOERR K.A., WOHLFIEL S.L. & ROBERTS G.D. (2003). Evaluation of the MicroSeq system for identification of mycobacteria by 16S ribosomal DNA sequencing and its integration into a routine clinical mycobacteriology laboratory. *J. Clin. Microbiol.,* **41**, 1447–1453.

HOOP R. (2002). *Mycobacterium tuberculosis* infection in a canary (*Serinus canaria* L.) and a blue-fronted Amazon parrot (*Amazona amazona aestiva*). *Avian Dis.*, **46**, 502–504.

INDERLIED C.B., KEMPER C.A. & BERMUDEZ L.E.M. (1993). The *Mycobacterium avium* complex. *Clin. Microbiol. Rev.,* **6**, 266–310.

KAEVSKA M., SLANA I., KRALIK P. & PAVLIK I. (2010). Examination *of Mycobacterium avium* subsp. *avium* distribution in naturally infected hens by culture and triplex quantitative real time PCR. *Veterinarni Medicina*, **55**, 325–330.

KAZDA J., PAVLIK I., FALKINHAM J. & HRUSKA K. (2009). The Ecology of *Mycobacteria*: Impact on Animal's and Human's Health, First Edition, Springer Science+Business Media BV, 520 pp. ISBN 978-1-4020-9412-5.

KUNZE Z.M., PORTAELS F. & MCFADDEN J.J. (1992). Biologically distinct subtypes of *Mycobacterium avium* differ in possession of insertion sequence IS*901*. *J. Clin. Microbiol.,* **30**, 2366–2372.

KIRSCHNER P., MEIER P.A. & BOTTGER E.C. (1993). Genotypic identification and detection of mycobacteria. *In:* Diagnostic Molecular Microbiology, Persing D.H., Smith T.F., Tenover F.C. & White T.C., eds. American Society for Microbiology, Washington DC, USA, 173–190.

LANTERI G., MARINO F., REALE S., VITALE F., MACRI F. & MAZZULLO G. (2011). *Mycobacterium tuberculosis* in a red-crowned parakeet (*Cyanoramphus novaezelandiae*). *J. Avian Med. Surg.,* **25**, 40–43.

MAES M., GIMÉNEZ J.F., D'ALESSANDRO A. & DE WAARD J.H. (2011). The stability of human, bovine and avian tuberculin purified protein derivative (PPD). *J. Infect. Dev. Ctries*, **5**, 781–785.

MIJS W., DE HAAS P., ROSSAU R., VAN DER LAAN T., RIGOUTS L., PORTAELS F. & VAN SOOLINGEN D. (2002). Molecular evidence to support a proposal to reserve the designation *Mycobacterium avium* subsp. *avium* to bird-type isolates and *M. avium* subsp. *hominissuis* for the human/porcine type of *M. avium. Int. J. Syst. Evol. Microbiol.,* **52**, 1505–1518.

MORAVKOVA M., HLOZEK P., BERAN V., PAVLIK I., PREZIUSO S., CUTERI V. & BARTOS M. (2008). Strategy for the detection and differentiation of *Mycobacterium avium* species in isolates and heavily infected tissues. *Res. Vet. Sci.,* **85**, 257–264.

NARSANA N., ALEJANDRA PEREZ M. & SUBRAMANIAN A. (2023). Mycobacteria in Organ Transplant Recipients. *Infect. Dis. Clin. North Am.*, S0891-5520(23)00040-5. doi: 10.1016/j.idc.2023.04.004.

~~PAVLIK I., MATLOVA L., DVORSKA L., BARTL J., OKTABCOVA L., DOCEKAL J. & PARMOVA I. (2003). Tuberculous lesions in pigs in the Czech Republic during 1990-1999: occurrence, causal factors and economic loses. *Veterinarni Medicina*, **48**, 113–125.~~

~~PAVLIK I., MATLOVA L., DVORSKA L., SHITAYE J. E. & PARMOVA I. (2005). Mycobacterial infections in cattle and pigs caused by *Mycobacterium avium* complex members and atypical mycobacteria in the Czech Republic during 2000–2004. *Veterinarni Medicina*, **50**, 281–290.~~

PAVLIK I., SVASTOVA P., BARTL J., DVORSKA L. & RYCHLIK I. (2000). Relationship between IS*901* in the *Mycobacterium avium* complex strains isolated from birds, animals, humans, and the environment and virulence for poultry. *Clin. Diagn. Lab. Immunol.,* **7**, 212–217.

PETERS M., PRODINGER W.M., GUMMER H., HOTZEL H., MOBIUS P. & MOSER I. (2007). *Mycobacterium tuberculosis* infection in a blue-fronted amazon parrot (*Amazona aestiva aestiva*). *Vet. Microbiol.*, **122**, 381–383.

POCKNELL A.M., MILLER B.J., NEUFELD J.L. & GRAHN B.H. (1996). Conjunctival mycobacteriosis in two emus (*Dromaius novaehollandiae*). *Vet. Pathol.*, **33**, 346–348.

REALINI L., DE RIDDER K., HIRSCHEL B. & PORTAELS F. (1999). Blood and charcoal added to acidified agar media promote the growth of *Mycobacterium genavense*. *Diagn. Microbiol. Infect. Dis.*, **34**, 45–50.

RIOJAS M.A., FRANK A.M., GREENFIELD S.R., KING S.P., MEEHAN C.J., STRONG M., WATTAM A.R. & HAZBÓN M.H. (2021). Identification and Characterization of Mycobacterial Species Using Whole-Genome Sequences. *Methods Mol. Biol.*, **2314**, 399–457.

RITACCO V., KREMER K., VAN DER LAAN T., PIJNENBURG J.E.M., DE HAAS P.E.W. & VAN SOOLINGEN D. (1998). Use of IS*901* and IS*1245* in RFLP typing of *Mycobacterium avium* complex: relatedness among serovar reference strains, human and animal isolates. *Int. J. Tuberculosis Lung Dis.*, **2**, 242–251.

ROZANSKA M. (1965). Preparation of antigen for whole blood rapid agglutination test and its specificity for diagnosis of avian tuberculosis. *Bull. Vet. Inst. Pulawy,* **9**, 20–25.

SAITO H., TOMIOKA H., SATO K., TASAKA H. & DAWSON D.J. (1990). Identification of various serovar strains of *Mycobacterium avium* complex by using DNA probes specific for *Mycobacterium avium* and *Mycobacterium intracellulare*. *J. Clin. Microbiol.,* **28**, 1694–1697.

SALAMATIAN I., GHANIEI A., MOSAVARI N., NOURANI H., KESHAVARZ R. & ESLAMPANAH M. (2020). Outbreak of avian mycobacteriosis in a commercial turkey breeder flock. *Avian Pathol.*, **49**, 296–304.

SATTAR A., ZAKARIA Z., ABU J., AZIZ S.A. & ROJAS-PONCE G. (2021). Isolation of Mycobacterium avium and other nontuberculous mycobacteria in chickens and captive birds in peninsular Malaysia. *BMC Vet. Res.*, **17**, 13.

SCHMIDT V., KOHLER H., HEENEMANN K. & MOBIUS P. (2022). Mycobacteriosis in Various Pet and Wild Birds from Germany: Pathological Findings, Coinfections, and Characterization of Causative Mycobacteria. *Microbiol. Spectr.,* 10(4): e0045222.

SCHMIDT V., SCHNEIDER S., SCHLOMER J., KRAUTWALD-JUNGHANNS M.E. & RICHTER E. (2008). Transmission of tuberculosis between men and pet birds: a case report. *Avian Pathol.*, **37**, 589–592.

SCHMITZ A., KORBEL R., THIEL S., WORLE B., GOHL C. & RINDER M. (2018a). High prevalence of Mycobacterium genavense within flocks of pet birds. *Vet. Microbiol.,* **218**, 40–44.

SCHMITZ A., RINDER M., THIEL S., PESCHEL A., MOSER K., REESE S. & KORBEL R. (2018b). Retrospective Evaluation of Clinical Signs and Gross Pathologic Findings in Birds Infected With *Mycobacterium genavense*. *J. Avian Med. Surg.*, **32**, 194–204.

SHITAYE J.E., GRYMOVA V., GRYM M., HALOUZKA R., HORVATHOVA A., MORAVKOVA M., BERAN V., SVOBODOVA J., DVORSKA-BARTOSOVA L. & PAVLIK I. (2009). *Mycobacterium avium* subsp. *hominissuis* infection in a pet parrot. *Emerg. Inf. Dis.,* **15**, 617–619.

SHITAYE J.E., HALOUZKA R., SVOBODOVA J., GRYMOVA V., GRYM M., SKORIC M., FICTUM P., BERAN V., SLANY M. & PAVLIK I. (2010). First isolation of *Mycobacterium genavense* in blue headed parrot (*Pionus menstruus*) imported from Surinam (South America) to the Czech Republic: a case report. *Veterinarni Medicina*, **55**, 339–347.

SHITAYE J.E., MATLOVA L., HORVATHOVA A., MORAVKOVA M., DVORSKA-BARTOSOVA L., TREML F., LAMKA J. & PAVLIK I. (2008). *Mycobacterium avium* subsp. *avium* distribution studied in a naturally infected hen flock and in the environment by culture, serotyping and IS*901* RFLP methods. *Vet. Microbiol.*, **127**, 155–164.

SHITAYE J.E., PARMOVA I., MATLOVA L., DVORSKA L., HORVATHOVA A., VRBAS V. & PAVLIK, I. (2006). Mycobacterial and *Rhodococcus equi* infections in pigs in the Czech Republic between the years 1996 and 2004: the causal factors and distribution of infections in the tissues. *Veterinarni Medicina,* **51**, 497–511.http://www.vri.cz/docs/vetmed/51-11-497.pdf

SLANA I., KAEVSKA M., KRALIK P., HORVATHOVA A. & PAVLIK, I. (2010). Distribution of *Mycobacterium avium* subsp. *avium* and *M. a. hominissuis* in artificially infected pigs studied by culture and IS*901* and IS*1245* quantitative real time PCR. *Vet. Microbiol.*, **144**, 437–443.

SOINI H., EEROLA E. & VILJANEN M.K. (1996). Genetic diversity among *Mycobacterium avium* complex Accu-Probe-positive isolates. *J. Clin. Microbiol.,* **34**, 55–57.

TELL L.A., FOLEY J., NEEDHAM M.L. & WALKER R.L. (2003a). Comparison of four rapid DNA extraction techniques for conventional polymerase chain reaction testing of three *Mycobacterium* spp. that affect birds. *Avian Dis.,* **47**, 1486–1490.

TELL L.A., LEUTENEGGER C.M., LARSEN R.S., AGNEW D.W., KEENER L., NEEDHAM M.L. & RIDEOUT B.A. (2003b). Real-time polymerase chain reaction testing for the detection of *Mycobacterium genavense* and *Mycobacterium avium* complex species in avian samples. *Avian. Dis.,* **47**, 1406–1415.

TELL L.A., WOODS L. & CROMIE R.L. (2001). Mycobacteriosis in birds. *Rev. Sci. Tech.,* **20**, 180–203.

THOREL M.F., HUCHZERMEYER H. & MICHEL A.L. (2001). *Mycobacterium avium* and *M. intracellulare* infection in mammals. *Rev. sci. tech. Off. int. Epiz.,* **20**, 204–218.

THOREL M.F., HUCHZERMEYER H., WEISS R. & FONTAINE J.J. (1997). *Mycobacterium avium* infections in animals. Literature review. *Vet. Res.,* **28**, 439–447.

THOREL M.F., KRICHEVSKY M. & LEVY-FREBAULT V.V. (1990). Numerical taxonomy of mycobactin-dependent mycobacteria, emended description of *Mycobacterium avium*, and description of *Mycobacterium avium* subsp. *avium* subsp. nov., *Mycobacterium avium* subsp. *paratuberculosis* subsp. nov., and *Mycobacterium avium* subsp. *silvaticum* subsp. nov. *Int. J. Syst. Bacteriol.*, **40**, 254–260.

TORTOLI E., MEEHAN C.J., GROTTOLA A., FREGNI SERPINI G., FABIO A., TROVATO A., PECORARI M. & CIRILLO D.M. (2019). Genome-based taxonomic revision detects a number of synonymous taxa in the genus *Mycobacterium*. *Infect. Genet. Evol.,* **75**, 103983.

VAN INGEN J., AL HAJOJ SAM., BOERE M., AL RABIAH F., ENAIMI M., DE ZWAAN R., TORTOLI E., DEKHUIJZEN R. & VAN SOOLINGEN D. (2009). *Mycobacterium riyadhense* sp. nov.; a non-tuberculous species identified as *Mycobacterium tuberculosis* by a commercial line-probe assay. *Int. J. Syst. Evol. Microbiol.,* **59**, 1049–1053.

~~VAN SOOLINGEN D., BAUER J., RITACCO V., CARDOSO LEAO S., PAVLI I., VINCENT V., RASTOGI N., GORI A., BODMER T., GARZELLI C. & GARCIA M.J. (1998). IS*1245* restriction fragment length polymorphism typing of *Mycobacterium avium* isolates: proposal for standardization. *J. Clin. Microbiol.*, **36**, 3051–3054.~~

WITTE C., FOWLER J.H., PFEIFFER W., HUNGERFORD L.L., BRAUN J., BURCHELL J., PAPENDICK R. & RIDEOUT B.A. (2021). Social network analysis and whole-genome sequencing to evaluate disease transmission in a large, dynamic population: A study of avian mycobacteriosis in zoo birds. *PLoS One*, **16**(6): e0252152.

WOLINSKY E. & SCHAEFER W.B. (1973). Proposed numbering scheme for mycobacterial serotypes by agglutination. *Int. J. Syst. Bacteriol.,* **23**, 182–183.

WORLD HEALTH ORGANIZATION (WHO) (1987). Requirements for Biological Substances No. 16, Annex 1: Requirement for Tuberculins. Technical Report Series No. 745, WHO, Geneva, Switzerland, 31–59.

*

* *

NB: There is currently (2024) no WOAH Reference Laboratory for avian tuberculosis
(please consult the WOAH Web site for the current list:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3http://www.oie.int/).

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

Annex 10. Chapter 3.4.1. 'Bovine anaplasmosis'

SECTION 3.4.

BOVINAE

CHAPTER 3.4.1.

BOVINE ANAPLASMOSIS

SUMMARY

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

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

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

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

Serological tests: *A competitive enzyme-linked immunosorbent assay (C-ELISA) has ~~been demonstrated to have~~ good sensitivity in detecting carrier animals. Card agglutination*

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

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

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

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

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

A. INTRODUCTION

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

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

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

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

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

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

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

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

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

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

~~the lowest biosafety level, equivalent to BSL1~~. Nevertheless the agent should be handled with appropriate biosafety and containment procedures as determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

B. DIAGNOSTIC TECHNIQUES

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

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

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
Agent id. = agent identification; CAT = card agglutination test; ~~CFT = complement fixation test;~~
C-ELISA = competitive enzyme-linked immunosorbent assay; ddasELISA = displacement double-antigen, sandwich ELISA;
IFAT = indirect fluorescent antibody test; PCR = polymerase chain reaction.
[(a)] See Appendix 1 of this chapter for justification table for the scores giving to the tests for this purpose.
[(b)] See Appendix 2 of this chapter for justification table for the scores giving to the tests for this purpose.
[(c)] See Appendix 3 of this chapter for justification table for the scores giving to the tests for this purpose.
[(d)] See Appendix 4 of this chapter for justification table for the scores giving to the tests for this purpose.
[(e)] See Appendix 5 of this chapter for justification table for the scores giving to the tests for this purpose.
[(f)] See Appendix 6 of this chapter for justification table for the scores giving to the tests for this purpose.
[(g)] A combination of agent identification methods applied on the same clinical sample is recommended.
[(h)] These tests do not distinguish infected from vaccinated animals.

1. Detection of the agent

1.1. Microscopic examination

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

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

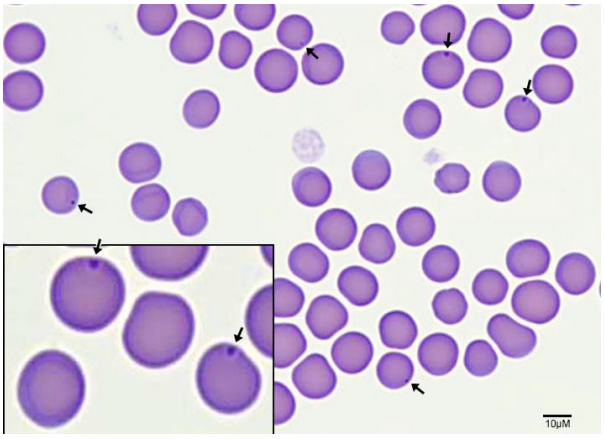


Figure 1. *Anaplasma marginale* ~~initial~~ inclusion bodies. A ~~Diff-Quick~~ stained blood smear from a bovine experimentally infected with *A. marginale*. Arrows point to the *A. marginale* ~~initial~~ inclusion bodies. Photo from S. Noh.

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

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

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

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

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

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

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

1.2. Polymerase chain reaction

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

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

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

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

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

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

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

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

Assay	Reference	Oligonucleotides[a]	Sequence 5'–3'[b]	Amplicon size (bp)	NCBI accession number
Real-time PCR	Carelli *et al.*, 2007	*Am_msp1b*_F	TTG-GCA-AGG-CAG-CAG-CTT	95	M59845
		*Am_msp1b*_R	TTC-CGC-GAG-CAT-GTG-CAT		
		*Am_msp1b*_PB	TCG-GTC-TAA-CAT-CTC-CAG-GCT-TTC-AT		
Real-time PCR	Futse *et al.*, 2003	*Am_msp5*_F	GCC-AAG-TGA-TGG-TGA-TAT-CGA	151	M93392
		*Am_msp5*_R	AGA-ATT-AAG-CAT-GTG-ACC-GCT-G		
		*Am_msp5*_PB	AAC-GTT-CAT-GTA-CCT-CAT-CAA		
Reverse-transcription real-time PCR	Reinbold *et al.*, 2010	*16S rRNA*_F[c]	CTC-AGA-ACG-AAC-GCT-GG	142	M60313
		*16S rRNA*_R[c]	CAT-TTC-TAG-TGG-CTA-TCC-C		
		*Am_16S rRNA*_PB[d]	CGC-AGC-TTG-CTG-CGT-GTA-TGG-T		
Real-time PCR[d]	Decaro *et al.*, 2008	*Ac_groEL*_F[e, f]	CTA-TAC-ACG-CTT-GCA-TCT-C	77	CP001759.1
		*Ac_groEL*_R[e, f]	CGC-TTT-ATG-ATG-TTG-ATG-C		
		*Ac_groEL*_PB[e, f]	TCA-TCA-TTC-TTC-CCC-TTT-ACC-TCG-T		

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

2. Serological tests

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

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

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

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

2.1. Competitive enzyme-linked immunosorbent assay

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

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

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

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

2.1.1. Kit reagents

A 96-well microtitre plate coated with rMSP5 antigen,

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

100×Mab–peroxidase conjugate,

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

Ready-to-use substrate and stop solutions,

Positive and negative controls

2.1.2. Test procedure

i) Add 70 µl of undiluted serum sample to the coated adsorption/transfer plate and incubate at room temperature for 30 minutes.

ii) Transfer 50 µl per well of the adsorbed undiluted serum to the rMSP5-coated plate and incubate at room temperature for 60 minutes.

ii) Discard the serum and wash the plate twice using diluted wash solution.

iii) Add 50 µl per well of the 1× diluted MAb–peroxidase conjugate to the rMSP5-coated plate wells, and incubate at room temperature for 20 minutes.

iv) Discard the 1×diluted MAb–peroxidase conjugate and wash the plate four times using diluted wash solution.

v) Add 50 µl per well of the substrate solution, cover the plate with foil, and incubate for 20 minutes at room temperature.

vi) Add 50 µl per well of stop solution to the substrate solution already in the wells and gently tap the sides of the plate to mix the wells.

vii) Immediately read the plate in the plate reader at 620, 630 or 650 nm.

2.1.3. Test validation

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

2.1.4. Interpretation of the results

The % inhibition is calculated as follows:

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

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

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

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

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

2.2. Indirect enzyme-linked immunosorbent assay

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

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

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

2.2.1. Test reagents

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

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

Blocking reagent (e.g. commercial dried skim milk)

Tris buffer 0.1 M, $MgCl_2$, 0.1 M, NaCl, 005 M, pH 9.8

Substrate *p*-Nitrophenyl phosphate disodium hexahydrate

Positive and negative controls.

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

i) Plates can be prepared ahead of time and kept under airtight conditions at –20°C.

ii) Carefully remove the plastic packaging before using plates, being careful not to touch the bottom of them as this can distort the optical density reading.

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

iv) For one plate, dissolve 1.1 g of skim milk (blocking agent) in 22 ml of PBST20.

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

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

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

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

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

x) Wash three times as described in point ~~subsection~~ vi.

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

xii) Remove the lid and wash three times as described in point vi above ~~make three washes with PBST20~~.

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

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

2.2.3. Data analysis

Analysis of results should take into account the following parameters.

i) The mean value of the blank wells.

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

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

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

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

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

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

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

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

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

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

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

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

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

2.3.1. Test reagents

i) A 96-well microtitre plate coated with either *A. marginale* or A. centrale recombinant protein

ii) PBS/Tween buffer (PBS (50mM sodium phosphate, 150 mM NaCL, pH 7.2) with 0.05% Tween-20)

iii) Blocking reagent (PBS with 10% commercial dried skim milk)

iv) Purified recombinant *A. marginale* MSP5 epitopes and *A. centrale* epitopes

v) Biotinylated recombinant *A. marginale* MSP5 epitopes and *A. centrale* epitopes

vi) Streptavidin-horse radish peroxidase (HRP) detection system

vii) Chromogenic substrate (1 mM 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt in0.05 M sodium citrate, pH 4.5, 0.0025% V/V H_2O_2 (100 µl/well).

viii) ELISA plate reader (405 nm reading)

ix) Positive and negative control sera for *A. marginale* and *A. centrale*

2.3.2. Test procedure

i) Plates are coated overnight.

ii) Block with blocking buffer for 1 hour at room temperature and wash three times with PBS/Tween buffer.

iii) Add undiluted serum 100 µl/well and incubate at 25°C for 1 hour at 100 rpm.

iv) Wash three times with PBS/Tween buffer.

v) Add 100 µl of *A. marginale* MSP5-biotin (1 µg/ml) plus *A. centrale* MSP5 (10 µg/ml) to *A. marginale* test wells. Add *A. centrale* MSP5-biotin (1 µg/ml) plus *A. marginale* MSP5 (10 µg/ml) in PBS/Tween buffer + 10% fat-free dried milk to *A. centrale* test wells.

vi) Incubate at 25°C for 1 hour at 100 rpm and wash the plate five times with PBS/Tween buffer.

vii) To detect the bound protein–biotin complex, add streptavidin-HRP diluted in 1/500 in PBS/Tween buffer with 10% dried milk for 1 hour at 25°C, 100 rpm.

vii) Wash five times with PBS/Tween buffer.

ix) Add chromogenic substrate based on manufacturer's instructions.

x) The reaction is measured by microplate reader spectrophotometer at 405 nm wavelength. The data are expressed in optical density (OD).

xi) OD_{405nm} <0.2 is considered negative.

xii) Results are expressed as the ratio between antibodies specific for *A. marginale* MSP5 and for *A. centrale* MSP5 (ODAm/ODAc). If the ratio is >0.38 the sample is considered positive for anti-*A. marginale* antibodies, and a ratio ≤ 0.38 is classified as vaccinated with *A. centrale*.

2.4. Card agglutination test

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

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

2.4.1. Test procedure

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

ii) On each circle of the test card (a clear perspex/plastic or glass plate marked with circles that are 18 mm in diameter), place next to each other, but not touching, 10 µl of bovine serum factor (BSF), 10 µl of test serum, and 5 µl of CAT antigen[1]. Negative and low positive control sera must be tested on each card.

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

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

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

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

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

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

~~2.4. Complement fixation test~~

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

2.5. Indirect fluorescent antibody test

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

2.6. Complement fixation test

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

C. REQUIREMENTS FOR VACCINES

1. Background

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

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

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

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

2. Outline of production and minimum requirements for conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

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

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

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

2.1.2. Quality criteria

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

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

2.2. Method of manufacture

2.2.1. Procedure

i) Production of frozen vaccine

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

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

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

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

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

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

ii) Production of chilled vaccine

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

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

iii) Use of vaccine

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

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

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

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

2.2.2. Requirements for substrates and media

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

2.2.3. In-process controls

i) Source and maintenance of vaccine donors

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

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

ii) Surgery

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

iii) Screening of vaccine donors before inoculation

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

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

iv) Monitoring of rickettsaemias following inoculation

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

v) Collection of blood for vaccine

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

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

vi) Dispensing of vaccine

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

2.2.4. Final product batch tests

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

i) Sterility and purity

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

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

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

ii) Safety

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

iii) Potency

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

2.3. Requirements for authorisation

2.3.1. Safety

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

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

2.3.2. Efficacy requirements

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

2.3.3. Stability

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

3. Vaccines based on biotechnology

There are no vaccines based on biotechnology available for anaplasmosis.

REFERENCES

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

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

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

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

BELL-SAKYI L., PALOMAR A.M., BRADFORD E.L. & SHKAP V. (2015). Propagation of the Israeli vaccine strain of *Anaplasma centrale* in tick cell lines. *Vet. Microbiol.,* **179**, 270–276.

BELLEZZE J., THOMPSON C.S., BOSIO A.S, TORIONI S.M. & PRIMO M.E. (2023). Development and field evaluation of an ELISA to differentiate *Anaplasma marginale*-infected from *A. centrale*-vaccinated cattle. *J. Vet. Diagn. Invest*., **35**, 204–208.

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

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

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

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

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

CHUNG C., WILSON C., BANDARANAYAKA-MUDIYANSELAGE C.-B., KANG E., ADAMS D.S., KAPPMEYER L.S., KNOWLES D.P., MCELWAIN T.F., EVERMANN J.F., UETI M.W., SCOLES G.A., LEE S.S. & MCGUIRE T.C. (2014). Improved diagnostic performance of a commercial *Anaplasma* antibody competitive enzyme-linked immunosorbent assay using recombinant major surface protein 5-glutathione S-transferase fusion protein as antigen. *J. Vet. Diagn. Invest.*, **26**, 61–71.

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

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

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

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

FUTSE J.E., UETI M.W., KNOWLES D.P. JR. & PALMER G.H. (2003). Transmission of *Anaplasma marginale* by *Boophilus microplus*: retention of vector competence in the absence of vector-pathogen interaction. *J. Clin. Microbiol.*. **41**, 3829–3834.

GONZALEZ, E. F., LONG R. F. & TODOROVIC R. A. (1978). Comparisons of the complement-fixation, indirect fluorescent antibody, and card agglutination tests for the diagnosis of bovine anaplasmosis. *Am. J. Vet. Res.,* **39**, 1538-1541.

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

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

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

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

KOCAN K. M., J. DE LA FUENTE, BLOUIN E. F., COETZEE J. F. & EWING S. A. (2010). The natural history of *Anaplasma marginale*. *Vet. Parasitol*., **167**, 95–107.

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

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

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

MATEI I.A., ESTRADA-PENA A., CUTLER S.J., VAYSSIER-TAUSSAT M., VARELA-CASTRO L., POTKONJAK A., ZELLER H. & MIHALCA A.D. (2019). A review on the eco-epidemiology and clinical management of human granulocytic anaplasmosis and its agent in Europe. *Parasit. Vectors,* **12,** 599.

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

MOLLOY J.B., BOWLES P.M., KNOWLES D.P., MCELWAIN T.F., BOCK R.E., KINGSTON T.G., BLIGHT G.W. & DALGLIESH R.J. (1999). Comparison of a competitive inhibition ELISA and the card agglutination test for

950 detection of antibodies to *Anaplasma marginale* and *Anaplasma centrale* in cattle. *Aust. Vet. J.,* **77**,
951 245–249.

952 NIELSEN K., SMITH P., GALL D., DE ESHAIDE S. T, WAGNER G. & DAJER A. (1996). Development and
953 validation of an indirect enzyme immunoassay for detection of antibody to *Anaplasma marginale* in
954 bovine sera. *Vet. Parasitol.*, **67**, 133–142.

955 NOH S. M. & BROWN W.C. (2012). Adaptive immune responses to infection and opportunities for vaccine
956 development (Anaplasmataceae). *Intracellular Pathogens II: Rickettsiales*. G. H. Palmer. Washington,
957 DC, USA, ASM Press. II: 330–365.

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

960 PIPANO E. (1995). Live vaccines against hemoparasitic diseases in livestock. *Vet. Parasitol.*, **57**, 213–
961 231.

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

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

966 RAMOS C.A., ARAUJO F.R., SANTOS R.C., MELO E.S., SOUSA L.C., VIDAL C.E., GUERRA N.R. & RAMOS R.A.
967 (2014). Development and assessment of a latex agglutination test based on recombinant MSP5 to
968 detect antibodies against *Anaplasma marginale* in cattle. *Braz. J. Microbiol.*, **45**, 199–204.

969 REINBOLD J.B., COETZEE J.F., HOLLIS L.C., NICKELL J.S., RIEGEL C.M., CHRISTOPHER J.A. & GANTA R.R.
970 (2010a). Comparison of iatrogenic transmission of *Anaplasma marginale* in Holstein steers via needle
971 and needle-free injection techniques. *Am. J. Vet. Res.*, **71**, 1178–1188.

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

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

977 SARLI M., THOMPSON C.S., NOVOA M., VALENTINI B.S., MASTROPAOLO M., ECHAIDE I.E., DE ECHAIDE S. T.
978 & PRIMO M.E. (2020). Development and evaluation of a double-antigen sandwich ELISA to identify
979 *Anaplasma marginale*-infected and *A. centrale*-vaccinated cattle. *J. Vet. Diagn. Invest.*, **32**, 70–76.

980 SHKAP V., KOCAN K., MOLAD T., MAZUZ M., LEIBOVICH B., KRIGEL Y., MICHOYTCHENKO A., BLOUIN E., DE LA
981 FUENTE J., SAMISH M., MTSHALI M., ZWEYGARTH E., FLEIDEROVICH E. L. & FISH L. (2009). Experimental
982 transmission of field *Anaplasma marginale* and the *A. centrale* vaccine strain by *Hyalomma excavatum*,
983 *Rhipicephalus sanguineus* and *Rhipicephalus* (*Boophilus*) annulatus ticks. *Vet. Microbiol.,* **134**, 254–
984 260.

985 SILAGHI C., NIEDER M., SAUTER-LOUIS C., KNUBBEN-SCHWEIZER G., PFISTER K. & PFEFFER M. (2018).
986 Epidemiology, genetic variants and clinical course of natural infections with *Anaplasma*
987 *phagocytophilum* in a dairy cattle herd. *Parasit. Vectors*, **11**, 20.

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

992 SILVA V.M., ARAUJO F.R., MADRUGA C.R., SOARES C.O., KESSLER R.H., ALMEIDA M.A., FRAGOSO S.P.,
993 SANTOS L.R., RAMOS C.A., BACANELLI G. & TORRES R.A. (2006). Comparison between indirect enzyme-

linked immunosorbent assays for *Anaplasma marginale* antibodies with recombinant Major Surface Protein 5 and initial body antigens. *Mem. Inst. Oswaldo Cruz.,* **101**, 511–516.

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

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

*
* *

NB: There is a WOAH Reference Laboratory for anaplasmosis (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 Laboratory for any further information on diagnostic tests, reagents and vaccines for bovine anaplasmosis

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

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

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

Appendix 2: Bovine anaplasmosis

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

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

[1] RLB is the reverse line blot test.

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

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

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

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Microscopic examination +++	Whole blood	No robust validation has been published.	N/A	N/A	1. Most laboratories have the capacity to make and examine blood smears. 2. *A. marginale* infected erythrocytes readily visible in clinically affected animals.	1. *A. marginale* colonies are small and can be difficult to differentiate from debris if animal has low rickettsemia. 2. Requires experience to identify *A. marginale* colonies. 3. Difficult to differentiate between *A. marginale* and *A. centrale*.	
PCR +++	Whole blood Various gene targets	Partial validation has been published.	51 cattle from 18 herds in three regions of southern Italy were tested by RLB[1] for *A. marginale A. centrale, A. bovis, T. buffeli, B bovis, A. phagocytophilum*, and *B. bigemina*. All cattle except 4 were positive for at least one of these pathogens.	See reference	Good reported concordance between nested PCR and real time PCR. High analytic sensitivity (10^1 DNA copies).	1. Must be performed in a lab equipped to extract DNA and have thermocyclers for real-time PCR. 2. Important to use PCR in conjunction with diagnosis of anaemia and blood smear because PCR can detect low level rickettsemia leading to misdiagnosis.	Carelli *et al.*, 2007

N/A: not available.

[1] RLB is the reverse line blot test.

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

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
CAT +	Serum Lysates of *A. marginale* isolated from red blood cells.	Reference test was blood smear. DSe 84.1[1]-100[2]% Dsp 97.9[1]-98.6[2]%	48 cattle raised in anaplasmosis free region. 82 animals from endemic region.[1] 86 sera from experimentally infected cattle and 183 sera from *A. marginale* free area[2]	See references	1. Can be done in field or in the laboratory	1. Antigen derived from infected cattle are difficult to produce and standardise. 2. May have false negative and false positive results. 3. Variation between tests depending on environmental conditions and the laboratory.	[1]Gonzalez *et al.*, 1978. [2]Molloy *et al.*, 1999.
C-ELISA +++ Bovine	Serum rMSP5-GST	Reference tests were nested PCR and IFAT. Dsp = 99.7% Dse = 100% 30% inhibition as determined by ROC analysis.	1. 358 known non-infected cattle from dairy herds maintained in tick free barns and no clinical history of clinical anaplasmosis. 2. 135 known positive sera as defined by nested PCR. 3. Intra-test comparison with 163 diagnostic samples with possible false positives based on rMSP5-GST C-ELISA. Test positive confirmation done with IFAT.	See reference	1. Updated version with improved specificity. 2. High sensitivity, detects persistently infected animals. 3. Commercially available. 4. Uses a standardised antigen. 5. Target antigen is highly conserved among *A. marginale* strains, thus detects infection with all strains of *A. marginale*. 6. Rapid.	1. Does not differentiate between infection with *A. marginale* and *A. centrale*. 2. May cross react with anti-*Ehrlichia* antibodies. 3. May not be readily available in all countries. 4. Requires a microplate absorbance reader. 5. Low percent of false positive results	Chung *et al.*, 2014.
IFAT++ Bovine	Serum Glass slides with RBCs infected with *A. marginale*	Reference test was blood. DSe 97.6% Dsp 89.6%	1. 48 cattle raised in anaplasmosis free region. 2. 82 animals from endemic region.	See references	1. Antigen is relatively easy to produce and store. 2. Does not require many reagents.	1.Relatively high false positive rate. 2. Time consuming and labour intensive so not suitable for high throughput. 3. Requires fluorescent microscope and blood smears with high rickettsemia.	Gonzalez *et al.*, 1978

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

Test with score and species	Sample type and target analytes	Accuracy	Test population used to measure accuracy	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
NA detection by (real-time) RT-PCR +++	Ear notch (skin), blood, milk	Performance has been demonstrated under field conditions in large control programs	Whole Swiss, German and Irish cattle populations	See references	- Very sensitive - Rapid - High-throughput - Well established internationally - Detects assay-dependent all BVDV species - Allows assay-dependent for differentiation of BVDV types 1 and 2 - Detects persistent and transient infection - Proficiency panel of different Pestivirus strains available - Detection of viral RNA in skin biopsy samples unaffected by maternally-derived antibodies, therefore allows for identification of persistently infected animals early in life - Successfully applied in ongoing or completed control programmes	- Possibility for contamination at sample collection or in laboratory, leading to false positive results - Needs specialised equipment - Detection of viral RNA does not imply per se that infectious virus is present	- Presi & Heim (2010). *Vet. Microbiol.*, **142**, 137–142 - Schweizer *et al.* (2021) *Front. Vet. Sci.*, **8**, 702730 - Wernike *et al.* (2017). *Pathogens*, **6** (4) - Graham *et al.* (2021) *Front. Vet. Sci.*, **8**, 674557
Antibody detection by ELISA +++	Bulk milk, blood	DSe and DSp differs based on the ELISA used (commercial/in-house) and the antibodies being tested (e.g. antibodies against structural (E2) and			- Simple to perform and cost-effective - Milk collection is non-invasive method with potential for herd screening with tank/bulk milk samples	- Some cross-reactivity with vaccines and other pestiviruses - PI animal will usually be seronegative	Beaudeau *et al.* (2001). *Vet. Microbiol.*, **80**, 329–337 Lanyon *et al.* (2013). *Aust. Vet. J.*, **91**, 52–56.

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

18 N/A: not available

Annex 11. Chapter 3.4.7. 'Bovine viral diarrhoea'

CHAPTER 3.4.7.

BOVINE VIRAL DIARRHOEA

SUMMARY

Cattle of all ages are susceptible to infection with bovine viral diarrhoea (BVD) viruses, including Pestivirus bovis (*commonly known as BVDV type 1* ~~(Pestivirus bovis)~~, Pestivirus tauri (*BVDV type 2* ~~(Pestivirus tauri)~~, *and* Pestivirus brazilense (*BVDV type 3* (~~Pestivirus brazilense)~~ (or *Hobi-like pestiviruses* ~~(type 3 [Pestivirus brazilense]~~). *Distribution is world-wide although some countries have recently eradicated the virus. BVDV infection results in a wide variety of clinical manifestations, including enteric and respiratory disease in any class of cattle, or reproductive and fetal disease following infection of a susceptible breeding female. Infection may be subclinical or extend to severe fatal disease. Animals that survive* in-utero *infection in the first trimester of gestation are almost always persistently infected (PI). PI animals provide the main reservoir of the virus in a population and excrete large amounts of virus in urine, faeces, discharges, milk and semen. Identification of such PI cattle is a key element in controlling the infection. It is important to avoid the trade of such animals. They may appear clinically healthy, or weak and unthrifty. Many PI animals die before reaching maturity. They may infrequently develop mucosal disease with anorexia, gastrointestinal erosions, and profuse diarrhoea, invariably leading to death. Mucosal disease can arise only in PI animals. Latent infections generally do not occur following recovery from acute infection. However, bulls may rarely have a prolonged and persistent testicular infection and excrete virus in semen for prolonged periods, perhaps indefinitely.*

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

Serological tests: *Acute infection with BVDV is best confirmed by demonstrating seroconversion using sequential paired samples, ideally from several animals in the group.*

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

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

A. INTRODUCTION

1. Impact of the disease

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

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

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

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

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

2. The causal agent

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

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

3. Pathogenesis

3.1. Acute infections

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

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

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

3.2. *In-utero* infections

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

3.3. Persistent infections

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

3.4. Mucosal disease

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

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

3.5. Semen and embryos

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

Biological materials used for *in-vitro* fertilisation techniques (bovine serum, bovine cell cultures) have a high risk of contamination and should be screened for BVDV. Incidents of apparent introduction of virus via such techniques have highlighted this risk. It is considered essential that serum supplements used in media should be free of contaminants as detailed in Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use,* using techniques described in Section B.~~3~~ 1.1 of this chapter.

4. Approaches to diagnosis and sample collection

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

4.1. Acute infections

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

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

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

4.2. Persistent infections

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

4.3. Mucosal disease

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

4.4. Reproductive materials

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

B. DIAGNOSTIC TECHNIQUES

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

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

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
ELISA = enzyme-linked immunosorbent assay; IHC = immunohistochemistry method; NA = nucleic acid; RT-PCR = reverse-transcription polymerase chain reaction; ~~ISH = *in-situ* hybridisation;~~ VN = virus neutralisation.

[(a)] See Appendix 1 of this chapter for justification table for the scores giving to the tests for this purpose.
[(b)] See Appendix 2 of this chapter for justification table for the scores giving to the tests for this purpose.
[(c)] See Appendix 3 of this chapter for justification table for the scores giving to the tests for this purpose.
[(d)] See Appendix 4 of this chapter for justification table for the scores giving to the tests for this purpose.
[(e)] See Appendix 5 of this chapter for justification table for the scores giving to the tests for this purpose.
[(f)] See Appendix 6 of this chapter for justification table for the scores giving to the tests for this purpose.
[(g)] A combination of agent detection methods applied on the same clinical sample is recommended.

1. Detection of the agent

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

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

1.1. Virus isolation

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

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

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

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

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

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

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

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

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

iii) The plate is incubated at 37°C for 4 days, either in a 5% CO_2 atmosphere or with the plate sealed.

iv) Each well is examined microscopically for evidence of cytopathology (cytopathic effect or CPE), or signs of cytotoxicity.

v) The cultures are frozen briefly at approximately –80°C and 50 µl of the culture supernatant is passaged to new cell cultures, repeating steps ~~3~~1.1.1.i to iv above.

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

- **Paraformaldehyde**

a) Add 200 µl of a 1/10 dilution of formaldehyde solution (approximately 3% concentration) to the plate and leave at room temperature for 10 minutes.

b) The contents of the plate are then discarded and the plate is washed.

c) Wash plates 5 times with 0.05% Tween 20 in water (an automatic microplate washer can be used with a low pressure and speed setting).

d) To each well add 50 µl of an antiviral antibody at the appropriate dilution (prepared in phosphate buffered saline/ PBS containing 1% gelatin) and incubate for 60–90 minutes at 37°C in a humidified chamber.

e) Wash plates five times as in step c).

f) Dilute the appropriate peroxidase conjugated antiserum to the optimum dilution in 1% gelatin/PBS (e.g. peroxidase conjugated rabbit anti-mouse immunoglobulin when the antiviral antibody is a mouse monoclonal). The optimum concentration should be determined for each batch of conjugate by “checkerboard” titration against reference positive and negative controls.

g) To each well of the microplate add 50 µl of the diluted peroxidase conjugate and incubate for 90 minutes at 37°C in a humidified chamber.

h) Wash plates five times as in step c).

i) “Develop” the plate by adding 3-amino-9-ethyl carbazole (AEC) substrate (100 µl/well) and allowing to react for 30 minutes at room temperature.

j) Add 100 µl of PBS to each well and add a lid to each plate.

k) Examine the wells by light microscopy, starting with the negative and positive control wells. There should be no or minimal staining apparent in the cells that were uninfected (negative control). The infected (positive control) cells should show a reddish- brown colour in the cytoplasm.

- **Acetone**

a) The plate is emptied by gentle inversion and rinsed in PBS.

b) The cells are fixed as follows: the plate is dipped into a bath of 20% acetone in PBS, emptied immediately and then transferred to a fresh bath of 20% acetone in PBS for 10 minutes. The plate is drained thoroughly and as much fluid as possible is removed by tapping and blotting. The plate is dried thoroughly for at least 3 hours at a temperature of 25–30°C (e.g. using radiant heat from a bench lamp). *Note:* the drying is part of the fixation process.

c) The fixed cells are rinsed by adding PBS to all wells.

d) The wells are drained and the antiviral BVD antibody (50 µl) is added to all wells at a predetermined dilution in PBS containing 1% Tween 80 (PBST) and 5% horse serum or 1% gelatin. (Horse serum or gelatin may be added to reduce nonspecific staining.)

e) Incubate at 37°C for 15 minutes.

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

g) Drain and add the appropriate anti-species serum conjugated to peroxidase at a predetermined dilution in PBST (50 µl per well) for 15 minutes at 37°C.

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

i) Rinse the plate in distilled water. Ensure all fluid is tapped out from the plate.

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

An alternative substrate can be made, consisting of 9 mg diaminobenzidine tetrahydrochloride and 6 mg sodium perborate tetrahydrate dissolved in 15 ml of PBS. Though the staining is not quite so intense, these chemicals have the advantage that they can be shipped by air.

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

Alternative methods for fixation of the cells may be used and include the use of heat (see Chapter 3.8.3 *Classical swine fever*, Section B.2.2.1.viii). These should be first evaluated to ensure that the capacity to detect viral antigen is not compromised.

1.1.2. Tube method for tissue or buffy coat suspensions

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

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

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

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

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

v) The culture should then be frozen and thawed for passage to fresh cultures for one or preferably two more passages (including the culture inoculated for the final immunostaining). At the final passage, after freeze–thaw the tissue culture fluid is harvested and passaged on to microtitre plates for culture and staining by the immunoperoxidase method (see section B.~~3~~1.1.1 above) or by the immunofluorescent method. For immunofluorescence, cover-slips are included in the tubes and used to support cultured cells. At the end of the culture period, the cover slips are removed, fixed in 100% acetone and stained with an immunofluorescent conjugate to BVDV. Examine the cover slips under a fluorescent microscope for diffuse, cytoplasmic fluorescence characteristic of pestiviruses. Alternatively, culture supernatant from the final passage can be screened by real-time RT-PCR (see below).

1.1.3. Virus isolation from semen

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

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

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

iii) Inoculate 1 ml of the semen/serum mixture into a monolayer of susceptible cells (see virus isolation from tissue above) in cell culture tubes or a six-well tissue culture plate.

iv) Incubate the cultures for 1 hour at 37°C.

v) Remove the mixture, wash the monolayer several times with maintenance medium and then add new maintenance medium to the cultures.

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

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

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

ix) At the end of the second passage, the supernatant from the freeze-thaw preparation should be passaged onto cultures in a suitable system for immunoperoxidase staining or other antigen detection or by real-time RT-PCR after

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

1.2. Nucleic acid detection

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

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

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

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

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

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

Real-time RT-PCR has been shown to be extremely useful to screen semen samples to demonstrate freedom from BVDV and, apart from speed, often gives superior results to virus isolation in cell culture, especially when low virus levels are present, such as may be found in bulls with a PTI. The real-time RT-PCR described here uses a pair of sequence-specific primers for amplification of target ~~D~~ RNA and a 5'-nuclease oligoprobe for the detection of amplified products. The oligoprobe is a single, sequence-specific oligonucleotide, labelled with two different fluorophores. The primers and probe are available commercially and several different fluorophores options are available. This pan-pestivirus real-time RT-PCR assay is designed to detect viral ~~D~~ RNA of all strains of BVDV types 1 (*Pestivirus bovis*) ~~and BVDV,~~ 2 (*Pestivirus tauri*) and 3 (*Pestivirus brazilense*) as well as ~~BDV,~~ CSFV (*Pestivirus suis*), some strains of BDV (*Pestivirus ovis*) and most atypical pestiviruses. The assay selectively amplifies a 208 base pair sequence of the 5' non-translated region (5' NTR) of the pestivirus genome. Details of the primers and probes are given in the protocol outlined below.

i) Sample preparation, equipment and reagents

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

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

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

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

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

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

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

ii) Extraction of RNA

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

iii) Real-time RT-PCR assay procedure

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

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

c) Primers and probe sequences

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

Forward: BVD 190-F 5'-GRA-GTC-GTC-ART-GGT-TCG-AC

Reverse: V326 5'-TCA-ACT-CCA-TGT-GCC-ATG-TAC

Probe: TQ-pesti 5'-FAM-TGC-YAY-GTG-GAC-GAG-GGC-ATG-C-TAMRA-3'

d) Preparation of reaction mixtures

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

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

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

e) Selection of controls

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

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

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

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

g) Real-time polymerase chain reaction

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

1× 48°C 10 minutes

1× 95°C 10 minutes

45 × (95°C 15 seconds, 60°C 1 minute)

h) Analysis of real-time PCR data

The software program is usually set to automatically adjust results by compensating for any background signal and the threshold level is usually set according to the manufacturer's instructions for the selected analysis software used. In this instance, a threshold is set at 0.05.

i) Interpretation of results

a) Test controls – all controls should give the expected results with positive controls (PC1 and PC2) falling within the designated range and both the negative control (NC) and no template control (NTC) should have no Ct values.

b) Test samples

1) Positive result: Any sample that has a cycle threshold (Ct) value less than 40 is regarded as positive.

2) Negative result: Any sample that shows no Ct value is regarded as negative. However, before reporting a negative result for a sample, the performance of the exogenous internal control should be checked and shown to give a result within the accepted range for that control (for example, a Ct value no more than 2–3 Ct units higher than the NTC).

1.3. Enzyme-linked immunosorbent assay for antigen detection

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

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

1.4. Immunohistochemistry

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

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

2. Serological tests

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

2.1. Virus neutralisation test

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

2.1.1. Test procedure

i) The test sera are heat-inactivated for 30 minutes at 56°C.

ii) From a starting dilution of 1/4, serial twofold dilutions of the test sera are made in a cell-culture grade flat-bottomed 96-well microtitre plate, using cell culture medium as diluent. For each sample, three or four wells are used at each dilution depending on the degree of precision required. At each dilution of serum, for each sample one well is left without virus to monitor for evidence of sample toxicity that could mimic viral cytopathology or interfere with virus replication. Control positive and negative sera should also be included in each batch of tests.

iii) An equal volume (e.g. 50 µl) of a stock of cytopathic strain of BVDV containing 100 $TCID_{50}$ (50% tissue culture infective dose) is added to each well. A back titration of virus stock is also done in some spare wells to check the potency of the virus (acceptance limits 30–300 $TCID_{50}$).

iv) The plate is incubated for 1 hour at 37°C.

v) A flask of suitable cells (e.g. bovine turbinate, bovine testis) is trypsinised and the cell concentration is adjusted to 1.5×10^5/ml. 100 µl of the cell suspension is added to each well of the microtitre plate.

vi) The plate is incubated at 37°C for 4–5 days, either in a 5% CO_2 atmosphere or with the plate sealed.

vii) The wells are examined microscopically for CPE or fixed and stained by immunoperoxidase staining using an appropriate monoclonal antibody. The VN titre for each serum is the dilution at which the virus is neutralised in 50% of the wells. This can be calculated by the Spearman–Kärber or Reed Muench methods. A seronegative animal will show no neutralisation at the lowest dilution (1/4), equivalent to a final dilution of 1/8. For accurate comparison of antibody titres, and particularly to demonstrate significant (more than fourfold) changes in titre, samples should be tested in parallel in the same test.

2.2. Enzyme-linked immunosorbent assay

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

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

2.2.1. Test procedure

i) Roller cultures of secondary calf testis cells with a high multiplicity of infection (about one), are inoculated with BVDV strain Oregon C24V, overlaid with serum-free medium and incubated for 24 hours at 37°C.

ii) The cells are scraped off and pelleted. The supernatant medium is discarded. The pellet is treated with two volumes of 2% OGP in PBS for 15 minutes at 4°C, and centrifuged to remove the cell debris. The supernatant antigen is stored in small aliquots at –70°C, or freeze-dried. Non-infected cells are processed in parallel to make a control antigen.

iii) The antigen is diluted to a predetermined dilution in 0.05 M bicarbonate buffer, pH 9.6. Alternate rows of an ELISA-grade microtitre plate are coated with virus and control antigens overnight at 4°C. The plates are then washed in PBS with 0.05% Tween 20 or Tween 80 (PBST) before use in the test.

iv) Test sera are diluted 1/50 in serum diluent (0.5 M NaCl; 0.01 M phosphate buffer; 0.05% Tween 20; 0.001 M ethylene diamine tetra-acetic acid; 1% polyvinyl pyrrolidone, pH 7.2) and added to virus- and control-coated wells for 1 hour at 37°C. The plates are then washed five times in PBST.

v) Rabbit anti-bovine IgG peroxidase conjugate is added at a predetermined dilution (in serum diluent) for 1 hour at 37°C, then the plates are again washed five times in PBST.

vi) A suitable enzyme substrate is added, such as hydrogen peroxide/tetramethyl benzidine. After colour development, the reaction is stopped with sulphuric acid and the absorbance is read on an ELISA plate reader. The value obtained with control antigen is subtracted from the test reaction to give a net absorbance value for each serum.

vii) It is recommended to convert net absorbance values to sample:positive ratio (or percentage positivity) by dividing net absorbance by the net absorbance on that test of a standard positive serum that has a net absorbance of about 1.0. This normalisation procedure leads to more consistent and reproducible results.

C. REQUIREMENTS FOR VACCINES

1. Background

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

1.1. Characteristics of a target product profile

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

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

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

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

2.1.1. Biological characteristics of the master seed

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

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

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

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

2.1.3. Validation as a vaccine strain

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

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

2.2. Method of manufacture

2.2.1. Procedure

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

2.2.2. Requirements for ingredients

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

2.2.3. In-process controls

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

2.2.4. Final product batch tests

i) Sterility

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in Chapter 1.1.9.

ii) Identity

Identity tests should demonstrate that no other strain of BVDV is present when several strains are propagated in a facility producing multivalent vaccines.

iii) Safety

Safety tests shall consist of detecting any abnormal local or systemic adverse reactions to the vaccine by all vaccination route(s). Batch-to-batch safety tests are required unless safety of the product is demonstrated and APPROVED in the registration dossier and production is consistent with that described in chapter 1.1.8.

The safety test is different to the inocuity test (see above).

Live vaccines must either be demonstrated to be safe in pregnant cattle (i.e. no transmission to the fetus), or should be licensed with a warning not to use them in pregnant animals. Live vaccines containing cytopathic strains should have an appropriate warning of the risk of inducing mucosal disease in PI cattle.

iv) Batch potency

BVD vaccines must be demonstrated to produce adequate immune responses, when used in their final formulation according to the manufacturer's published instructions. The minimum quantity of infectious virus and/or antigen required to produce an acceptable immune response should be determined. *In-vitro* assays should be used to monitor individual batches during production.

2.3. Requirements for authorisation/registration/licensing

2.3.1. Manufacturing process

For registration of a vaccine, all relevant details concerning manufacture of the vaccine and quality control testing should be submitted to the relevant authorities. Unless otherwise specified by the authorities, information should be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

There is no standard method for the manufacture of a BVD vaccine, but conventional laboratory techniques with stationary, rolled or suspension (micro-carriers) cell cultures may be used. Inactivated vaccines can be prepared by conventional methods, such as binary ethylenimine or beta-propiolactone inactivation (Park & Bolin, 1987). A variety of adjuvants may be used.

2.3.2. Safety requirements

In-vivo tests should be undertaken using a single dose, overdose (for live vaccines only) and repeat doses (taking into account the maximum number of doses for primary vaccination and, if appropriate, the first revaccination/booster vaccination) and contain the maximum permitted antigen load and, depending on the formulation of the vaccine, the maximum number of vaccine strains.

i) Target and non-target animal safety

The safety of the final product formulation of both live and inactivated vaccines should be assessed in susceptible young calves that are free of maternally derived antibodies and in pregnant cattle. They should be checked for any local reactions following administration, and, in pregnant cattle, for any effects on the unborn calf.

Live attenuated vaccines may contribute to immunosuppression that might increase mortality. It may also contribute to the development of mucosal disease in PI animals that is an animal welfare concern. Therefore vaccination of PI animals with live attenuated vaccines containing cytopathic BVDV should be avoided. Live attenuated vaccines must not be capable of being transmitted to other unvaccinated animals that are in close contact.

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

Virus seeds that have been passaged at least up to and preferably beyond the passage limit specified for the seed should be inoculated into young calves to confirm that there is no evidence of disease. If a live attenuated vaccine has been registered for use in pregnant animals, reversion to virulence tests should also include pregnant animals. Live attenuated vaccines should not be transmissible to unvaccinated 'in-contact' animals.

iii) Precautions (hazards)

BVDV is not considered to be a human health hazard. Standard good microbiological practice should be adequate for handling the virus in the laboratory. A live virus vaccine should be identified as harmless for people administering the product. However adjuvants included in either live or inactivated vaccines may cause injury to people. Manufacturers should provide adequate warnings that medical advice should be sought in the case of self-injection (including for adjuvants, oil-emulsion vaccine, preservatives, etc.) with warnings included on the product label/leaflet so that the vaccinator is aware of any danger.

2.3.3. Efficacy requirements

The potency of the vaccine should be determined by inoculation into seronegative and virus negative calves, followed by monitoring of the antibody response. Antigen content can be assayed by ELISA and adjusted as required to a standard level for the particular vaccine. Standardised assay protocols applicable to all vaccines do not exist. Live vaccine batches may be assayed by infectivity titration. Each production batch of vaccine should undergo potency and safety testing as batch release criteria. BVD vaccines must be demonstrated to produce adequate immune responses, as outlined above, when used in their final formulation according to the manufacturer's published instructions.

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

To date, there are no commercially available vaccines for BVDV that support use of a true DIVA strategy. Experimental subunit vaccines based on baculovirus-expressed BVD viral glycoprotein E2 have been described but are not available commercially. They offer a future prospect of 'marker vaccines' when used in connection with a complementary serological test. Experimental BVDV E2 DNA vaccines and BVDV E2 subunit vaccines expressed using transgenic plants and alphavirus replicon or chimeric pestivirus vaccines have also been described.

2.3.5. Duration of immunity

There are few published data on the duration of antibody following vaccination with a commercial product. Protocols for their use usually recommend a primary course of two inoculations and boosters at yearly intervals. Only limited data are available on the antibody levels that correlate with protection against respiratory infections (Bolin & Ridpath, 1995; Howard *et al*., 1989) or *in-utero* infection (Brownlie *et al*., 1995). However, there are many different commercial formulations and these involve a range of adjuvants that may support different periods of efficacy. Consequently, duration of immunity data must be generated separately for each commercially available product by undertaking challenge tests at the end of the period for which immunity has been claimed.

2.3.6. Stability

There are no accepted guidelines for the stability of BVD vaccines, but it can be assumed that attenuated virus vaccine (freeze-dried) should remain potent for at least 1 year if kept at 4°C. Inactivated virus vaccine could have a longer shelf life at 4°C. Lower temperatures could prolong shelf life for either type, but adjuvants in killed vaccine may preclude this. Bulk antigens that have not been formulated into finished vaccine can be reliably stored frozen at low temperatures but the antigen quality should be monitored with *in-vitro* assays prior to incorporation into a batch of vaccine.

REFERENCES

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

BAUERMANN F.V., FLORES E.F. & RIDPATH J.F. (2012). Antigenic relationships between bovine viral diarrhea virus 1 and 2 and HoBi virus: possible impacts on diagnosis and control. *J. Vet. Diagn. Invest.* (official publication of the American Association of Veterinary Laboratory Diagnosticians), **24**, 253–261.

BAUERMANN F.V., HARMON A., FLORES E.F., FALKENBERG S.M., REECY J.M. & RIDPATH J.F. (2013). *In vitro* neutralization of HoBi-like viruses by antibodies in serum of cattle immunized with inactivated or modified live vaccines of bovine viral diarrhea viruses 1 and 2. *Vet. Microbiol.*, **166**, 242–245.

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

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

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

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

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

CHEN M., LIU M., LIU S. & SHANG Y. (2021). HoBi-like pestivirus infection leads to bovine death and severe respiratory disease in China. *Transbound. Emerg. Dis.*, **68**, 1069–1074.

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

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

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

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

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

FUX R. & WOLF G. (2012). Transient elimination of circulating bovine viral diarrhoea virus by colostral antibodies in persistently infected calves: a pitfall for BVDV-eradication programs? *Vet. Microbiol*., **161**, 1.

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

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

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

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

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

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

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

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

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

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

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

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

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

POSTLER T.S., BEER M., BLITVICH B.J., BUKH J., DE LAMBALLERIE X., DREXLER J.F., IMRIE A., KAPOOR A., KARGANOVA G.G., LEMEY P., LOHMANN V., SIMMONDS P., SMITH D.B., STAPLETON J.T. & KUHN J.H. (2023). Renaming of the genus Flavivirus to Orthoflavivirus and extension of binomial species names within the family Flaviviridae. *Arch. Virol.*, **168**, DOI: 10.1007/s00705-023-05835-1

READ A.J., GESTIER S., PARRISH K., FINLAISON D.S., GU X., O'CONNOR T.W. & KIRKLAND P.D. (2020). Prolonged Detection of Bovine Viral Diarrhoea Virus Infection in the Semen of Bulls. *Viruses*, **12**, 674. doi: 10.3390/v12060674. PMID: 32580423; PMCID: PMC7354483.

SCHWEIZER M., STALDER H., HASLEBACHER A., GRISIGER M., SCHWERMER H. & DI LABIO E. (2021). Eradication of bovine viral diarrhoea (BVD) in cattle in Switzerland: Lessons taught by the complex biology of the virus. *Front. Vet. Sci.,* **8**, 702730.

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

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

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

*

* *

NB: There are WOAH Reference Laboratories for bovine viral diarrhoea (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 bovine viral diarrhoea

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2015.

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

Test with score and species	Sample type and target analytes	Accuracy	Test population used to measure accuracy	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
NA detection by (real-time) RT-PCR +++	Ear notch (skin), blood, milk	Performance has been demonstrated under field conditions in large control programs	Whole Swiss, German and Irish cattle populations	See references	- Very sensitive - Rapid - High-throughput - Well established internationally - Detects assay-dependent all BVDV species - Allows assay-dependent for differentiation of BVDV types 1 and 2 - Detects persistent and transient infection - Proficiency panel of different Pestivirus strains available - Detection of viral RNA in skin biopsy samples unaffected by maternally-derived antibodies, therefore allows for identification of persistently infected animals early in life - Successfully applied in ongoing or completed control programmes	- Possibility for contamination at sample collection or in laboratory, leading to false positive results - Needs specialised equipment - Detection of viral RNA does not imply per se that infectious virus is present	- Presi & Heim (2010). *Vet. Microbiol.*, **142**, 137–142 - Schweizer *et al.* (2021) *Front. Vet. Sci.*, **8**, 702730 - Wernike *et al.* (2017). *Pathogens*, **6** (4) - Graham *et al.* (2021) *Front. Vet. Sci.*, **8**, 674557
Antibody detection by ELISA +++	Bulk milk, blood	DSe and DSp differs based on the ELISA used (commercial/in-house) and the antibodies being tested (e.g. antibodies against structural (E2) and non-structural (NS2-3) proteins.			- Simple to perform and cost-effective - Milk collection is non-invasive method with potential for herd screening with tank/bulk milk samples - Bulk milk sensitive indicator for PI in herd	- Some cross-reactivity with vaccines and other pestiviruses - PI animal will usually be seronegative - Bulk milk from herd excludes males, non-lactating or young stock	Beaudeau *et al.* (2001). *Vet. Microbiol.*, **80**, 329–337 Lanyon *et al.* (2013). *Aust. Vet. J.*, **91**, 52–56.

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

3 N/A: not available

Appendix 2: Bovine viral diarrhoea

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

Test with score and species	Sample type and target analytes	Accuracy	Test population used to measure accuracy	Validation Report	Advantages: expert opinion	Disadvantages: expert opinion	References
Virus isolation ++	Serum, whole blood.	Considered reference test; DSe <90% compared with real-time RT-PCR; DSp ~100%	N/A	Historical information with no formal validation	- High degree of specificity - Identifies presence of infectious virus	- Requires specialised cell culture capabilities and access to BVDV free materials - Reduced sensitivity in presence of MDA (diagnostic gap); takes several weeks for maximum DSe	Edmonson *et al.* (2007); Toker & Yesilbag (2021)
Antigen detection by ELISA +++	Serum, whole blood, skin biopsy (e.g. ear notch)	DSe 67–100% and DSp 98.8–100% relative to virus isolation have been reported but usually DSe and DSp are extremely high			Relatively simple to perform, rapid, can be cost-effective and suitable for high-throughput applications. There is no need for cell culture facility.	Maternal antibodies in colostrum may interfere with testing for antigen in serum using ELISA in calves. Ear notch samples are less affected. PI calves *in utero* defies detection.	Zimmer *et al.* (2004). *Vet. Microbiol.*, **100**, 145–149
NA detection by (real-time) RT-PCR +++	Ear notch (skin), blood; nasal or oral swab	- Depending on the assay analytical sensitivity of less than 10 genome copies/reaction - Accuracy 98.73% based on a proficiency test panel consisting of five ear notch and five serum samples		See references	- Very sensitive - Rapid - High-throughput - Well established internationally - Depending on the assay detects all BVDV species - Allows assay-dependent for differentiation of BVDV types 1 and 2 - Detects persistent and transient infection - Proficiency panel of different Pestivirus strains available - Detection of viral RNA in skin biopsy samples unaffected by maternally-derived antibodies	- Possibility for contamination at sample collection or in laboratory, leading to false positive results - Needs specialised equipment	- Hoffmann *et al.* (2006). *J. Virol. Methods*, **136**, 200–209. - Wernike *et al.* (2019). *Vet. Microbiol.*, **239**, 108452.

Test with score and species	Sample type and target analytes	Accuracy	Test population used to measure accuracy	Validation Report	Advantages: expert opinion	Disadvantages: expert opinion	References
Virus neutralisation test ++	Serum	DSe & DSp both extremely high, both >99%. Historical reference serological test.	N/A	Historical information with no formal validation	Very high specificity - Negative results generally indicate that an animal has not been infected provided it has been tested for virus/antigen Animals that give positive results have usually cleared the infection provided that they are not pregnant. Bulls present a special situation. If they seroconvert during a period of quarantine, there is a risk that there will be virus in their semen. Further, a small proportion of seropositive bulls may have a persistent testicular infection that may last for many months. To ensure freedom from infection, semen must be tested on several occasions using real-time RT-PCR. Virus isolation is not sufficiently sensitive and the presence of virus may be masked by antibodies.	- ASe can vary depending on virus strain used - Requires cell culture, good quality samples - time consuming to perform, takes 5 days to obtain results - Labour intensive - Due to the biology of the virus (birth of PI calves that are not able to amount a specific immune response to the virus strain they are infected with) antibody-negative animals could be PI (in non-BVDV-free populations)	N/A
Antibody detection by ELISA ++	Blood, Individual milk sample	DSe and DSp differs based on the ELISA used (commercial/in-house) and the antibodies being tested (e.g. antibodies against structural (E2) and non-structural (NS2-3) proteins.			- Simple to perform and cost-effective - Milk collection is non-invasive method - Paired samples can be used to confirm acute infection. - Ensure milk is collected directly from the teat rather than bulk-milk tank to ensure no cross contamination/false-positives	- Maternal antibodies in colostrum may interfere with testing for antibodies in serum using ELISA in calves. Calves should be tested after 9 months of age after maternal antibodies have waned. - PI animal will be seronegative and may impact receiving herds if moved. - Using milk, limited to lactating cow only	N/A

6 N/A: not available

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

Test with score and species	Sample type and target analytes	Accuracy	Test population used to measure accuracy	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
Antigen detection by ELISA +++	Serum, whole blood, skin biopsy	DSse 67%–100% and DSp 98.8–100% reported			Relatively simple to perform, rapid, can be cost-effective and suitable for high-throughput applications. There is no need for cell culture facility.	Maternal antibodies in colostrum may interfere with testing for antigen in serum using ELISA in calves. Ear notch samples are less affected. PI calves *in utero* defy detection.	Zimmer *et al.* (2004). *Vet. Microbiol.*, **100**, 145–149
NA detection by (real-time) RT-PCR +++	Ear notch (skin), blood; milk; nasal or oral swab	Utility has been demonstrated under field conditions in large control programs	Whole Swiss, German and Irish cattle populations	See references	- Very sensitive - Rapid - High-throughput - Well established internationally - Depending on assay, detects all BVDV species - Allows assay-dependent differentiation of BVDV types 1 and 2 - Detects persistent and transient infection - Proficiency panel of different Pestivirus strains available - Detection of viral RNA in skin biopsy samples unaffected from maternally-derived antibodies, therefore allows for identification of persistently infected animals early in life - Successfully applied in ongoing or completed control programmes (see references)	- Possibility for contamination at sample collection or in laboratory, leading to false positive results - Needs specialised equipment	- Presi & Heim (2010). *Vet. Microbiol.*, **142**, 137–142 - Schweizer *et al.* (2021). *Front. Vet. Sci.*, **8**, 702730 - Wernike *et al.* (2017). *Pathogens*, **6** (4) - Graham *et al.* (2021). *Front. Vet. Sci.*, **8**, 674557

Test with score and species	Sample type and target analytes	Accuracy	Test population used to measure accuracy	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
Antibody detection by ELISA ++	Bulk milk, Blood	DSe and DSp differs based on the ELISA used (commercial/in-house) and the antibodies being tested (e.g. antibodies against structural (E2) and non-structural (NS2-3) proteins.			- Simple to perform and cost-effective - Milk collection is non-invasive method - Can be used on a population basis to monitor freedom from infection by selection of appropriate aged animals e.g. borne after eradication achieved; for dairy herds by testing tank milk to monitor lactating animals	- Some cross-reactivity with antibodies from vaccines and other pestiviruses - PI animal will be seronegative - Bulk milk from herd does not include males, non-lactating or young stock	Laureyns *et al.* (2010)
Virus isolation ++	Serum, whole blood	Considered reference test ; DSe <90% compared with real-time RT-PCR; DSp ~100%	N/A	Historical information with no formal validation	- High degree of specificity - Identifies presence of infectious virus. - used to confirm the status of difficult cases and to provide isolates for intensive analysis e.g. NA sequencing	- Requires specialised cell culture capabilities and access to BVDV free materials - Reduced sensitivity in presence of MDA (diagnostic gap)	N/A
Virus neutralisation test ++	Serum	DSe & DSp both extremely high, both >99%. Historical reference serological test.	N/A	Historical information with no formal validation	Very high specificity - Used for confirming the virus free status of a population after eradication; - Used as a confirmatory test when surveillance utilises an ELISA	- ASe can vary depending on virus strain used - Requires cell culture, good quality samples - Takes 5 days to obtain results	N/A

9 N/A: not available

Appendix 4: Bovine viral diarrhoea
Intended purpose of test: confirmation of clinical cases

Test with score and species	Sample type and target analytes	Accuracy	Test population used to measure accuracy	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
Virus isolation ++	Serum, whole blood, tissue extracts	Considered reference test; DSe <90% compared with real-time RT-PCR; DSp ~100%	Not available	Historical information with no formal validation	- High degree of specificity - Identifies presence of infectious virus - Preferred method to identify presence of cytopathogenic strains and hence confirmation of mucosal disease - Provides virus isolates for detailed characterisation	- Requires specialised cell culture capabilities and access to BVDV free materials - Reduced sensitivity in presence of maternally derived antibodies (diagnostic gap) -Requires high quality samples to avoid bacterial contamination	– Meyling (1984)
Antigen detection by ELISA +++	Serum, whole blood, skin biopsy	DSe 67%–100% and DSp 98.8% to 100% reported			Relatively simple to perform, rapid, can be cost-effective and suitable for high-throughput applications. There is no need for cell culture facility.	Maternal antibodies in colostrum may interfere with testing for antigen in serum using ELISA in calves. Ear notch samples are less affected. PI calves *in utero* defies detection.	
NA detection by (real-time) RT-PCR +++	Blood; nasal, oral or conjunctival swab (in cases of respiratory disease) or faecal swab (enteric disease)	Depending on the assay analytical sensitivity of less than 10 genome copies/reaction		See reference	- Very sensitive - Rapid - High-throughput - Well established internationally - Depending on the assay, detects all BVDV species - Allows assay-dependent for differentiation of BVDV types 1 and 2 - Detects persistent and transient infection - Proficiency panel of different Pestivirus strains available - Detection of viral RNA in skin biopsy samples unaffected from maternally derived antibodies	- Possibility for contamination at sample collection or in laboratory, leading to false positive results - Needs specialised equipment	- Hoffmann ***et al.*** (2006). *J. Virol. Methods*, **136**, 200–209.
Antigen detection by IHC ++	Fixed tissues or frozen sections for Ag detection or NA if using ISH	Lower DSe than other methods; high DSp	N/A	N/A	Allows visualisation of viral components in lesions and assessment of tissue distribution	Technically demanding, limited reagents for detection of Ag in formalin-fixed tissues	
Antibody detection by ELISA +	Paired serum samples, fetal fluids (blood,	DSe and DSp may differ depending on the ELISA used (commercial/in-			- Simple to perform and cost-effective.	- Some cross-reactivity with antibodies induced by other pestiviruses.	

Test with score and species	Sample type and target analytes	Accuracy	Test population used to measure accuracy	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
	pericardial, thoracic	house) and the antibodies being tested (e.g. antibodies against structural (E2) and non-structural (NS2-3) proteins.			- Can be used to differentiate between acute and persistent infections by demonstration of seroconversion in acute infections - Detection of antibodies in aborted fetuses, stillborn animals can confirm *in utero* infection in second half of gestation	- PI animals are usually seronegative (in both of the paired samples)	

12 N/A: not available

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

Test with score and species	Sample type and target analytes	Accuracy	Test population used to measure accuracy	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
Antigen detection by ELISA +++	Serum, whole blood	DSe 67–100% and DSp 98.8–100% reported			Relatively simple to perform, rapid, very cost-effective and suitable for high-throughput applications. There is no need for cell culture facility.	Maternal antibodies in colostrum may interfere with testing for antigen in serum using ELISA in calves. Ear notch samples are less affected. PI calves *in utero* defies detection.	Sarrazin *et al.* (2013). *Prev. Vet. Med.*, **108**, 28–37
NA detection by (real-time) RT-PCR +++	Ear notch (skin), blood, milk		Whole Swiss, German and Irish cattle populations	See references	- Very sensitive - Rapid - High-throughput - Well established internationally - Depending on the assay, detects all BVDV species - Allows assay-dependent for differentiation of BVDV types 1 and 2 - Detects persistent and transient infection - Proficiency panel of different Pestivirus strains available - Detection of viral RNA in skin biopsy samples unaffected from maternally-derived antibodies	- Possibility for contamination at sample collection or in laboratory, leading to false positive results - Needs specialised equipment	- Presi & Heim (2010). *Vet. Microbiol.*, **142**, 137–142 - Schweizer *et al.* (2021). *Front. Vet. Sci.*, **8**, 702730 - Wernike *et al.* (2017) *Pathogens*, **6** (4) - Graham *et al.* (2021). F*ront. Vet. Sci.*, **8**, 674557
Antibody detection by ELISA +++	Bulk milk, blood	DSe and DSp may differdepending on the ELISA used (commercial/in-house) and the antibodies being tested (e.g. antibodies against structural (E2) and non-structural (NS2-3) proteins.			- Simple to perform and cost-effective - Milk collection is non-invasive method	- Some cross-reactivity with antibodies induced by vaccines and other pestiviruses. - PI animal will be seronegative - Bulk milk from herd excludes males, non-lactating or young stock.	Barrett *et al.* (2022) *BMC Vet Res.*, 18, 210.
Virus neutralisation test +	Serum	DSe & DSp both extremely high, both >99%. Historical reference serological test.	N/A	Historical information with no formal validation	- Very high specificity - Allows differentiation of antibodies to BVDV species	- ASe can vary depending on virus strain used - Requires cell culture, good quality samples - Takes 5 days to obtain results. Labour intensive - not amenable to testing very large numbers of samples,	N/A

Test with score and species	Sample type and target analytes	Accuracy	Test population used to measure accuracy	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
						- No differentiation between infected and vaccinated animals	

15 N/A: not available

Appendix 6: Bovine viral diarrhoea

Intended purpose of test: immune status in individual animals or populations (post-vaccination)

Test with score and species	Sample type and target analytes	Accuracy	Test population used to measure accuracy	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
Antibody detection by ELISA +++	Individual milk, bulk milk, blood (antibodies present against structural and non-structural proteins)	DSe and DSp differs based on the ELISA used (commercial/in-house) and the antibodies being tested (e.g. antibodies against structural (E2) and non-structural (NS2-3) proteins.			- Simple to perform and cost-effective - Milk collection is non-invasive method	- Some cross-reactivity with antibodies induced by vaccines and other pestiviruses. While a DIVA capability is preferred, this is very difficult achieve using the combinations of existing vaccines and serological assays. Live attenuated vaccines preclude a DIVA capability unless genetically modified to include a 'marker' gene or deletion that can be detected by a serological assay. - PI animal will be seronegative - Bulk milk from herd excludes males, non-lactating or young stock	Raue *et al.* (2011). *Vet. J.*, 187, 330–334; Gonzalez *et al.*, (2014). *Vet J.*, 199, 424–428. Sayers *et al.*, (2015). *Vet. J.*, 205, 56–61.
Virus neutralisation test +++	Serum	DSe & DSp both extremely high, both >99%. Historical reference serological test.	N/A	Historical information with no formal validation	- Very high specificity - Good correlation with immunity - Can provide a measure of cross protection between BVDV species	- ASe can vary depending on virus strain used - Requires cell culture, good quality samples - Labour intensive, takes 5 days to obtain results - No differentiation between infected and vaccinated animals	N/A

N/A: not available

Annex 12. Chapter 3.4.12 ‘Lumpy skin disease’

CHAPTER 3.4.12.

LUMPY SKIN DISEASE

SUMMARY

Description of the disease: *Lumpy skin disease (LSD) is a poxvirus disease of cattle characterised by fever, nodules on the skin, mucous membranes and internal organs, emaciation, enlarged lymph nodes, oedema of the skin, and sometimes death. The disease is of economic importance as it can cause a temporary reduction in milk production, temporary or permanent sterility in bulls, damage to hides and, occasionally, death. Various strains of capripoxvirus are responsible for the disease. These are antigenically indistinguishable from strains causing sheep pox and goat pox yet distinct at the genetic level. LSD has a partially different geographical distribution from sheep and goat pox, suggesting that cattle strains of capripoxvirus do not infect and transmit between sheep and goats. Transmission of LSD virus (LSDV) is thought to be predominantly by arthropods, natural contact transmission in the absence of vectors being inefficient. Lumpy skin disease is endemic in most ~~many~~ African and Middle Eastern countries. Between 2012 and 2022, LSD spread into south-east Europe, the Balkans, Russia and Asia as part of the Eurasian LSD epidemic.*

Pathology: *the nodules are firm and may extend to the underlying subcutis and muscle. Acute histological key lesions consist of epidermal vacuolar changes with intracytoplasmic inclusion bodies and dermal vasculitis. Chronic key histological lesions consist of fibrosis and necrotic sequestrae.*

Detection of the agent: *Laboratory confirmation of LSD is most rapid using a real-time or conventional polymerase chain reaction (PCR) method specific for capripoxviruses in combination with a clinical history of a generalised nodular skin disease and enlarged superficial lymph nodes in cattle. Ultrastructurally, capripoxvirus virions are distinct from those of parapoxvirus, which causes bovine papular stomatitis and pseudocowpox, but cannot be distinguished morphologically from orthopoxvirus virions, including cowpox and vaccinia viruses, both of which can cause disease in cattle, although neither causes generalised infection and both are uncommon in cattle. LSDV will grow in tissue culture of bovine, ovine or caprine origin. In cell culture, LSDV causes a characteristic cytopathic effect and intracytoplasmic inclusion bodies that is distinct from infection with Bovine herpesvirus 2, which causes pseudo-lumpy skin disease and produces syncytia and intranuclear inclusion bodies in cell culture. Capripoxvirus antigens can be demonstrated in tissue culture using immunoperoxidase or immunofluorescent staining and the virus can be neutralised using specific antisera.*

A variety of conventional and real-time PCR tests as well as isothermal amplification tests using capripoxvirus-specific primers have been published for use on a variety of samples.

Serological tests: *The virus neutralisation test (VNT) and enzyme-linked immunosorbent assays (ELISAs) are widely used and have been validated. The agar gel immunodiffusion test and indirect immunofluorescent antibody test are less specific than the VNT due to cross-reactions with antibody to other poxviruses. Western blotting using the reaction between the P32 antigen of LSDV with test sera is both sensitive and specific, but is difficult and expensive to carry out.*

Requirements for vaccines: *All strains of capripoxvirus examined so far, whether derived from cattle, sheep or goats, are antigenically similar. Attenuated cattle strains, and strains derived from sheep and goats have been used as live vaccines against LSDV.*

A. INTRODUCTION

Lumpy skin disease (LSD) was first seen in Zambia in 1929, spreading into Botswana by 1943 (Haig, 1957), and then into South Africa the same year, where it affected over eight million cattle causing major economic loss. In 1957 it entered Kenya, ~~at the same time as~~ associated with an outbreak of sheep pox (Weiss, 1968). In 1970 LSD spread north into the Sudan, by 1974 it had spread west as far as Nigeria, and in 1977 was reported from Mauritania, Mali, Ghana and Liberia. Another epizootic of LSD between 1981 and 1986 affected Tanzania, Kenya, Zimbabwe, Somalia and the Cameroon, with reported mortality rates in affected cattle of 20%. The occurrence of LSD north of the Sahara desert and outside the African continent was confirmed for the first time in Egypt and Israel between 1988 and 1989, and was reported again in 2006 (Brenner *et al.,* 2006). In the past decade, LSD occurrences have been reported in the Middle Eastern, European and Asian regions (for up-to-date information, consult WOAH WAHIS interface[1]). Lumpy skin disease outbreaks tend to be sporadic, depending upon animal movements, immune status, and wind and rainfall patterns affecting vector populations. The principal method of transmission is thought to be mechanical by various arthropod vectors (Tuppurainen *et al*., 2015).

Lumpy skin disease virus (LSDV) belongs to the family *Poxviridae*, subfamily *Chordopoxvirinae* ~~*Chordopoxviridae*~~, and genus *Capripoxvirus*. In common with other poxviruses LSDV replicates in the cytoplasm of an infected cell, forming distinct perinuclear viral factories. The LSD virion is large and brick-shaped measuring 293–299nm (length) and 262–273nm (width). The LSDV genome structure is also similar to other poxviruses, consisting of double-stranded linear DNA that is 25% GC-rich, approximately 150,000 bp in length, and encodes around 156 open reading frames (ORFs). An inverted terminal repeat sequence of 2200–2300 bp is found at each end of the linear genome. The linear ends of the genome are joined with a hairpin loop. The central region of the LSDV genome contains ORFs predicted to encode proteins required for virus replication and morphogenesis and exhibit a high degree of similarity with genomes of other mammalian poxviruses. The ORFs in the outer regions of the LSDV genome have lower similarity and likely encode proteins involved in viral virulence and host range determinants.

Phylogenetic analysis shows the majority of LSDV strains group into two monophyletic clusters (cluster 1.1 and 1.2) (Biswas *et al.,* 2020; Van Schalkwyk *et al.,* 2021). Cluster 1.1 consists of LSDV Neethling vaccine strains that are based on the LSDV/Neethling/LW-1959 vaccine strain (Kara *et al.,* 2003; Van Rooyen *et al.,* 1959; van Schalkwyk *et al.,* 2020) and historic wild-type strains from South Africa. Cluster 1.2 consists of wild-type strains from southern Africa, Kenya, the northern hemisphere, and the Kenyan KSGP O-240 commercial vaccine. In addition to these two clusters, there have recently been recombinant LSDV strains isolated from clinical cases of LSD in the field in Russia and central Asia (Flannery *et al.,* 2021; Sprygin *et al.,* 2018; 2020; Wang *et al.,* 2021). These recombinant viruses show unique patterns of accessory gene alleles, consisting of sections of both wild-type and “vaccine” LSDV strains.

The severity of the clinical signs of LSD is highly variable and depends on a number of factors, including the strain of capripoxvirus, the age of the host, immunological status and breed. *Bos taurus* is generally more susceptible to clinical disease than *Bos indicus;* the Asian buffalo (*Bubalus spp.*) has also been reported to be susceptible. Within *Bos taurus,* the fine-skinned Channel Island breeds develop more severe disease, with lactating cows appearing to be the most at risk. However, even among groups of cattle of the same breed kept together under the same conditions, there is a large variation in the clinical signs presented, ranging from subclinical infection to death (Carn & Kitching, 1995). There may be failure of the virus to infect the whole group, probably depending on the virulence of the virus isolate, immunological status of the host, host genotype~~,~~ and vector prevalence. Seroprevalence studies, experimental infections and case reports have provided indications that several wildlife species (e.g. springbok, impala, giraffe, camel, banteng) are susceptible to LSDV infection (Dao *et al.,* 2022; Hedger & Hamblin, 1983; Kumar *et al.,* 2023; Porco *et al.,* 2023). The scarcity of documented outbreaks in wildlife and the fact that available studies remain limited in number and mostly involve only a few animals, make it difficult to determine the role of wildlife in LSDV epidemiology. This topic deserves further study,

[1] https://www.woah.org/en/what-we-do/animal-health-and-welfare/disease-data-collection/

especially given the current spread of LSDV in new geographical areas where large numbers of naïve, potentially susceptible wild bovines and other ruminants are present.

The incubation period under field conditions has not been reported, but following experimental inoculation is 6–9 days until the onset of fever. In the acutely infected animal, there is an initial pyrexia, which may exceed 41°C and persist for 1 week. All the superficial lymph nodes become enlarged. In lactating cattle there is a marked reduction in milk yield. Lesions develop over the body, particularly on the head, neck, udder, scrotum, vulva and perineum between 7 and 19 days after virus inoculation (Coetzer, 2004). The characteristic integumentary lesions are multiple, well circumscribed to coalescing, 0.5–5 cm in diameter, firm, flat-topped papules and nodules. The nodules involve the dermis and epidermis, and may extend to the underlying subcutis and occasionally to the adjacent striated muscle. These nodules have a creamy grey to white colour on cut section, which may initially exude serum, but over the ensuing 2 weeks a cone-shaped central core or sequestrum of necrotic material/necrotic plug ("sit-fast") may appear within the nodule. The acute histological lesions consist of epidermal vacuolar changes with intracytoplasmic inclusion bodies and dermal vasculitis. The inclusion bodies are numerous, intracytoplasmic, eosinophilic, homogenous to occasionally granular and they may occur in endothelial cells, fibroblasts, macrophages, pericytes, and keratinocytes. The dermal lesions include vasculitis with fibrinoid necrosis, oedema, thrombosis, lymphangitis, dermal-epidermal separation, and mixed inflammatory infiltrate. The chronic lesions are characterised by an infarcted tissue with a sequestered necrotic core, often rimmed by granulation tissue gradually replaced by mature fibrosis. At the appearance of the nodules, the discharge from the eyes and nose becomes mucopurulent, and keratitis may develop. Nodules may also develop in the mucous membranes of the mouth and alimentary tract, particularly the abomasum and in the trachea and the lungs, resulting in primary and secondary pneumonia. The nodules on the mucous membranes of the eyes, nose, mouth, rectum, udder and genitalia quickly ulcerate, and by then all secretions, ocular and nasal discharge and saliva contain LSD virus (LSDV). The limbs may be oedematous and the animal is reluctant to move. Pregnant cattle may abort, and there is a report of intrauterine transmission (Rouby & Aboulsoudb, 2016). Bulls may become permanently or temporarily infertile and the virus can be excreted in the semen for prolonged periods (Irons *et al*., 2005). Recovery from severe infection is slow; the animal is emaciated, may have pneumonia and mastitis, and the necrotic plugs of skin, which may have been subject to fly strike, are shed leaving deep holes in the hide (Prozesky & Barnard, 1982).

The main differential diagnosis is pseudo-LSD caused by bovine herpesvirus 2 (BoHV-2). This is usually a milder clinical condition, characterised by superficial nodules, resembling only the early stage of LSD. Intra-nuclear inclusion bodies and viral syncytia are histopathological characteristics of BoHV-2 infection not seen in LSD. Other differential diagnoses (for integumentary lesions) include: dermatophilosis, dermatophytosis, bovine farcy, photosensitisation, actinomycosis, actinobacillosis, urticaria, insect bites, besnoitiosis, nocardiosis, demodicosis, onchocerciasis, pseudo-cowpox, and cowpox. Differential diagnoses for mucosal lesions include: foot and mouth disease, bluetongue, mucosal disease, malignant catarrhal fever, infectious bovine rhinotracheitis, and bovine papular stomatitis.

LSDV is not transmissible to humans. However, all laboratory manipulations must be performed at an appropriate containment level determined using 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 LSD and their purpose*

Method	**Purpose**					
	Population freedom from infection	**Individual animal freedom from infection prior to movement**	**Contribute to eradication policies**	**Confirmation of clinical cases**	**Prevalence of infection – surveillance**	**Immune status in individual animals or populations post-vaccination**
Detection of the agent						
Virus isolation	+	++	+	+++	+	–

Method	Purpose					
	Population freedom from infection	**Individual animal freedom from infection prior to movement**	**Contribute to eradication policies**	**Confirmation of clinical cases**	**Prevalence of infection – surveillance**	**Immune status in individual animals or populations post-vaccination**
PCR	++	+++	++	+++	+	–
TEM	–	–	–	+	–	–
Detection of immune response						
VNT	++	++	++	++	++	++
IFAT	+	+	+	+	+	+
ELISA	++	++	++	++	++	++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; TEM = Transmission electron microscopy; VNT = virus neutralisation test;
IFAT = indirect fluorescent antibody test; ELISA = enzyme-linked immunosorbent assay.

1. Detection of the agent

1.1. Specimen collection, submission and preparation

Material for virus isolation and antigen detection should be collected as biopsies or from skin nodules at post-mortem examination. Samples for virus isolation should preferably be collected within the first week of the occurrence of clinical signs, before the development of neutralising antibodies (Davies, 1991; Davies *et al*., 1971), however virus can be isolated from skin nodules for at least 3–4 weeks thereafter. Samples for genome detection using conventional or real-time polymerase chain reaction (PCR) may be collected when neutralising antibody is present. Following the first appearance of the skin lesions, the virus can be isolated for up to 35 days and viral nucleic acid can be demonstrated via PCR for up to 3 months (Tuppurainen *et al*., 2005; Weiss, 1968). Buffy coat from blood collected into heparin or EDTA (ethylene diamine tetra-acetic acid) during the viraemic stage of LSD (before generalisation of lesions or within 4 days of generalisation), can also be used for virus isolation. Samples for histology should include the lesion and tissue from the surrounding (non-lesion) area, be a maximum size of 2 cm^3, and be placed immediately following collection into ten times the sample volume of 10% neutral buffered formal saline.

Tissues in formalin have no special transportation requirements in regard to biorisks. Blood samples with anticoagulant for virus isolation from the buffy coat should be placed immediately on ice after gentle mixing and processed as soon as possible. In practice, the samples may be kept at 4°C for up to 2 days prior to processing, but should not be frozen or kept at ambient temperatures. Tissues for virus isolation and antigen detection should be kept at 4°C, on ice or at –20°C. If it is necessary to transport samples over long distances without refrigeration, the medium should contain 10% glycerol; the samples should be of sufficient size (e.g. 1 g in 10 ml) that the transport medium does not penetrate the central part of the biopsy, which should be used for virus isolation.

~~Samples for histology should include the lesion and tissue from the surrounding (non-lesion) area, be a maximum size of 2 cm^3, and be placed immediately following collection into ten times the sample volume of 10% neutral buffered formaldehyde. Tissues in formalin have no special transportation requirements in regard to biorisks.~~ Material for histology should be prepared using standard techniques and stained with haematoxylin and eosin (H&E) (Burdin, 1959). Lesion material for virus isolation and antigen detection is minced using a sterile scalpel blade and forceps and then macerated in a sterile steel ball-bearing mixer mill, or ground with a pestle in a sterile mortar with sterile sand and an equal volume of sterile phosphate buffered saline (PBS) or serum-free modified Eagle's medium containing sodium penicillin (1000 international units [IU]/ml), streptomycin sulphate

(1 mg/ml), mycostatin (100 IU/ml) or fungizone (amphotericin, 2.5 µg/ml) and neomycin (200 IU/ml). The suspension is freeze–thawed three times and then partially clarified using a bench centrifuge at 600 ***g*** for 10 minutes. In cases where bacterial contamination of the sample is expected (such as when virus is isolated from skin samples), the supernatant can be filtered through a 0.45 µm pore size filter after the centrifugation step. Buffy coats may be prepared from unclotted blood using centrifugation at 600 ***g*** for 15 minutes, and the buffy coat carefully removed into 5 ml of cold double-distilled water using a sterile Pasteur pipette. After 30 seconds, 5 ml of cold double-strength growth medium is added and mixed. The mixture is centrifuged at 600 ***g*** for 15 minutes, the supernatant is discarded and the cell pellet is suspended in 5 ml growth medium, such as Glasgow's modified Eagle's medium (GMEM). After centrifugation at 600 ***g*** for a further 15 minutes, the resulting pellet is suspended in 5 ml of fresh GMEM. Alternatively, the buffy coat may be separated from a heparinised sample by using a Ficoll gradient.

1.2. Virus isolation on cell culture

LSDV will grow in tissue culture of bovine, ovine or caprine origin. MDBK (Madin–Darby bovine kidney) cells are often used, as they support good growth of the virus and are well characterised (Fay *et al.,* 2020). Primary cells, such as lamb testis (LT) cells also support viral growth, but care needs to be taken to ensure they are not contaminated with viruses such as bovine viral diarrhoea virus. One ml of clarified supernatant or buffy coat is inoculated onto a confluent monolayer in a 25 cm^2 culture flask at 37°C and allowed to adsorb for 1 hour. The culture is then washed with warm PBS and covered with 10 ml of a suitable medium, such as GMEM, containing antibiotics and 2% fetal calf serum. If available, tissue culture tubes containing appropriate cells and a flying cover-slip, or tissue culture microscope slides, are also infected.

The flasks/tissue culture tubes are examined daily for 7–14 days for evidence of cytopathic effects (CPE). Infected cells develop a characteristic CPE consisting of retraction of the cell membrane from surrounding cells, and eventually rounding of cells and margination of the nuclear chromatin. At first only small areas of CPE can be seen, sometimes as soon as 2 days after infection; over the following 4–6 days these expand to involve the whole cell monolayer ~~sheet~~. If no CPE is apparent by day 14, the culture should be freeze–thawed three times, and clarified supernatant inoculated on to a fresh cell monolayer. At the first sign of CPE in the flasks, or earlier if a number of infected cover-slips are being used, a cover-slip should be removed, fixed in acetone and stained using H&E. Eosinophilic intracytoplasmic inclusion bodies, which are variable in size but up to half the size of the nucleus and surrounded by a clear halo, are diagnostic for poxvirus infection. PCR may be used as an alternative to H&E for confirmation of the diagnosis. The CPE can be prevented or delayed by adding specific anti-LSDV serum to the medium. In contrast, the herpesvirus that causes pseudo-LSD produces a Cowdry type A intranuclear inclusion body. It also forms syncytia.

An ovine testis cell line (OA3.T~~s~~) has been evaluated for the propagation of capripoxvirus isolates (Babiuk *et al.*, 2007), however this cell line has been found to be contaminated with pestivirus and should be used with caution.

1.3. Polymerase chain reaction (PCR)

The conventional gel-based PCR method described below is a simple, fast and sensitive method for the detection of capripoxvirus genome in EDTA blood, semen or tissue culture samples (Tuppurainen *et al*., 2005).

1.3.1. Test procedure

The extraction method described below can be replaced using commercially available DNA extraction kits.

i) Freeze and thaw 200 µl of blood in EDTA, semen or tissue culture supernatant and suspend in 100 µl of lysis buffer containing 5 M guanidine thiocyanate, 50 mM potassium chloride, 10 mM Tris/HCl (pH 8); and 0.5 ml Tween 20.

ii) Cut skin and other tissue samples into fine pieces using a sterile scalpel blade and forceps. Grind with a pestle in a mortar. Suspend the tissue samples in 800 µl of the above mentioned lysis buffer.

iii) Add 2 µl of proteinase K (20 mg/ml) to blood samples and 10 µl of proteinase K (20 mg/ml) to tissue samples. Incubate at 56°C for 2 hours or overnight, followed by heating at 100°C for 10 minutes. Add phenol:chloroform:isoamylalcohol (25:24:1 [v/v]) to the samples in a 1:1 ratio. Vortex and incubate at room temperature for 10 minutes. Centrifuge the samples at 16,060 ***g*** for 15 minutes at 4°C. Carefully collect the upper, aqueous phase (up to 200 µl) and transfer into a clean 2.0 ml tube. Add two volumes of ice cold ethanol (100%) and 1/10 volume of 3 M sodium acetate (pH 5.3). Place the samples at –20°C for 1 hour. Centrifuge again at 16,060 ***g*** for 15 minutes at 4°C and discard the supernatant. Wash the pellets with ice cold 70% ethanol (100 µl) and centrifuge at 16,060 ***g*** for 1 minute at 4°C. Discard the supernatant and dry the pellets thoroughly. Suspend the pellets in 30 µl of nuclease-free water and store immediately at –20°C (Tuppurainen *et al*., 2005). Alternatively a column-based extraction kit may be used.

iv) The primers for this PCR assay were developed from the gene encoding the viral attachment protein. The size of the expected amplicon is 192 bp (Ireland & Binepal, 1998). The primers have the following gene sequences:

Forward primer 5'-TCC-GAG-CTC-TTT-CCT-GAT-TTT-TCT-TAC-TAT-3'

Reverse primer 5'-TAT-GGT-ACC-TAA-ATT-ATA-TAC-GTA-AAT-AAC-3'.

v) DNA amplification is carried out in a final volume of 50 µl containing: 5 µl of 10 × PCR buffer, 1.5 µl of $MgCl_2$ (50 mM), 1 µl of dNTP (10 mM), 1 µl of forward primer, 1 µl of reverse primer, 1 µl of DNA template (~10 ng), 0.5 µl of *Taq* DNA polymerase and 39 µl of nuclease-free water. The volume of DNA template required may vary and the volume of nuclease-free water must be adjusted to the final volume of 50 µl.

vi) Run the samples in a thermal cycler as follows: 2 minutes at 95°C; then 45 seconds at 95°C, 50 seconds at 50°C and 1 minute at 72°C (34 cycles); 2 minutes at 72°C and hold at 4°C until analysis.

vii) Mix 10 µl of each sample with loading dye and load onto a 1.5% agarose gel in TAE buffer (Tris/acetate buffer containing EDTA). Load a parallel lane with a 100 bp DNA-marker ladder. Electrophoretically separate the products using approximately 8–10 V/cm for 40–60 minutes and visualise with a suitable DNA stain and transilluminator.

Quantitative real-time PCR methods have been described that are reported to be faster and have higher sensitivity than conventional PCRs (Balinsky *et al*., 2008; Bowden *et al*., 2008). A real-time PCR method that differentiates between LSDV, sheep pox virus and goat pox virus has been published (Lamien *et al.,* 2011).

Quantitative real-time PCR assays have been designed to differentiate between Neethling-based LSDV strains, which are often used for vaccination, and wild-type LSDV strains from cluster 1.2 (Agianniotaki *et al.,* 2017; Pestova *et al.,* 2018; Vidanovic *et al.,* 2016). These "DIVA" assays (DIVA: differentiation of infected from vaccinated animals) enable, for example, differentiation of "Neethling response" caused by vaccination with a LSDV Neethling vaccine strain from disease caused by infection with a cluster 1.2 wild-type virus. However these DIVA PCR assays cannot distinguish between a LSDV Neethling vaccine strain and the novel recombinant LSDV strains ~~recently~~ isolated from disease outbreaks in Asia (~~Byadovskaya *et al.,* 2021;~~ Flannery *et al.,* 2021). These DIVA assays are also not capable of discriminating between LSDV Neethling vaccine strains and recently characterised (historic) wild-type viruses from South Africa belonging within cluster 1.1 (Van Schalkwyk *et al.,* 2020; 2021). Consequently, in regions where recombinant strains (currently Asia and possibly elsewhere) or wild-type cluster 1.1 strains are circulating (currently South Africa and possibly elsewhere), these DIVA assays are not suitable for distinguishing vaccine and wild-type virus. Thus, in order to overcome these constraints, whole genome sequencing is recommended.

1.4. Transmission electron microscopy

The characteristic poxvirus virion can be visualised using a negative staining preparation technique followed by examination with an electron microscope. There are many different negative staining protocols, an example of which is given below.

1.4.1. Test procedure

Before centrifugation, material from the original biopsy suspension is prepared for examination under the transmission electron microscope by floating a 400-mesh hexagonal electron microscope grid, with pioloform-carbon substrate activated by glow discharge in pentylamine vapour, onto a drop of the suspension placed on parafilm or a wax plate. After 1 minute, the grid is transferred to a drop of Tris/EDTA buffer, pH 7.8, for 20 seconds and then to a drop of 1% phosphotungstic acid, pH 7.2, for 10 seconds. The grid is drained using filter paper, air-dried and placed in the electron microscope. The capripox virion is brick shaped, covered in short tubular elements and measures approximately 290 × 270 nm. A host-cell-derived membrane may surround some of the virions, and as many as possible should be examined to confirm their appearance (Kitching & Smale, 1986).

The ~~capripox~~ virions of capripoxvirus are indistinguishable from those of orthopoxvirus, but, apart from vaccinia virus and cowpox virus, which are both uncommon in cattle and do not cause generalised infection, no other orthopoxvirus is known to cause lesions in cattle. However, vaccinia virus may cause generalised infection in young immunocompromised calves. In contrast, orthopoxviruses are a common cause of skin disease in domestic buffalo (*Bubalus bubalis*) causing buffalo pox, a disease that usually manifests as pock lesions on the teats, but may cause skin lesions at other sites, such as the perineum, the medial aspects of the thighs and the head. Orthopoxviruses that cause buffalo pox cannot be readily distinguished from capripoxvirus by electron microscopy. The virions of parapoxvirus ~~virions~~ that cause bovine papular stomatitis and pseudocowpox are smaller, oval in shape and each is covered in a single continuous tubular element that appears as striations over the virion. Capripoxvirus virions are also distinct from the herpesvirus that causes pseudo-LSD (also known as “Allerton” or bovine herpes mammillitis).

1.5. Fluorescent antibody tests

Capripoxvirus antigen can be identified on infected cover-slips or tissue culture slides using fluorescent antibody tests. Cover-slips or slides should be washed and air-dried and fixed in cold acetone for 10 minutes. The indirect test using immune cattle sera is subject to high background colour and nonspecific reactions. However, a direct conjugate can be prepared from sera from convalescent cattle (or from sheep or goats convalescing from capripox) or from rabbits hyperimmunised with purified capripoxvirus. Uninfected tissue culture should be included as a negative control as cross-reactions can cause problems due to antibodies to cellular components (pre-absorption of these from the immune serum helps solve this issue).

1.6. Immunohistochemistry

Immunohistochemistry using F80G5 monoclonal antibody specific for capripoxvirus ORF 057 has been described for detection of LSDV antigen in the skin of experimentally infected cattle (Babiuk *et al.,* 2008).

1.7. Isothermal genome amplification

Molecular tests using loop-mediated isothermal amplification to detect capripoxvirus genomes are reported to provide sensitivity and specificity similar to real-time PCR with a simpler method and lower cost (Das *et al.,* 2012; Murray *et al.,* 2013). Field validation of the Das *et al.* method was reported by Omoga *et al.* (2016).

2. Serological tests

All the viruses in the genus *Capripoxvirus* share a common major antigen for neutralising antibodies and it is thus not possible to distinguish strains of capripoxvirus from cattle, sheep or goats using serological techniques.

2.1. Virus neutralisation

A test serum can either be titrated against a constant titre of capripoxvirus (100 $TCID_{50}$ [50% tissue culture infective dose]) or a standard virus strain can be titrated against a constant dilution of test serum in order to calculate a neutralisation index. Because of the variable sensitivity of tissue culture to capripoxvirus, and the consequent difficulty of ensuring the accurate and repeatable seeding of 100 $TCID_{50}$/well, the neutralisation index is the preferred method in most laboratories, although it does require a larger volume of test sera. The test is described using 96-well flat-bottomed tissue-culture grade microtitre plates, but it can be performed equally well in tissue culture tubes with the appropriate changes to the volumes used, although it is more difficult to read an end-point in tubes.

2.1.1. Test procedure

i) Test sera, including a negative and a positive control, are diluted 1/5 in Eagle's/HEPES (N-2-hydroxyethylpiperazine, N-2-ethanesulphonic acid) buffer and inactivated at 56°C for 30 minutes.

ii) Next, 50 µl of the first inactivated serum is added to columns 1 and 2, rows A to H of the microtitre plate. The second serum is placed in columns 3 and 4, the third in columns 5 and 6, the positive control serum is placed in columns 7 and 8, the negative control serum is placed in columns 9 and 10, and 50 µl of Eagle's/HEPES buffer (without serum) is placed in columns 11 and 12, and to all wells in row H.

iii) A reference strain of capripoxvirus, usually a vaccine strain known to grow well in tissue culture, with a titre of over $\log_{10}$ 6 $TCID_{50}$ per ml is diluted in Eagle's/HEPES in bijoux bottles to give a log dilution series of $\log_{10}$ 5.0, 4.0, 3.5, 3.0, 2.5, 2.0, 1.5 $TCID_{50}$ per ml (equivalent to $\log_{10}$ 3.7, 2.7, 2.2, 1.7, 1.2, 0.7, 0.2 $TCID_{50}$ per 50 µl).

iv) Starting with row G and the most diluted virus preparation, 50 µl of virus is added to each well in that row. This is repeated with each virus dilution, the highest titre virus dilution being placed in row A.

v) The plates are covered and incubated for 1 hour at 37°C.

vi) An appropriate cell suspension (such as MDBK cells) is prepared from pregrown monolayers as a suspension of 10^5 cells/ml in Eagle's medium containing antibiotics and 2% fetal calf serum. Following incubation of the microtitre plates, 100 µl of cell suspension is added to all the wells, except wells H11 and H12, which serve as control wells for the medium. The remaining wells of row H are cell and serum controls.

vii) The microtitre plates are covered and incubated at 37°C for 9 days.

viii) Using an inverted microscope, the monolayers are examined daily from day 4 for evidence of CPE. There should be no CPE in the cells of row H. Using the 0240 KSGP vaccine strain of capripoxvirus, by way of example, the final reading is taken on day 9, and the titre of virus in each duplicate titration is calculated using the Kärber method. If left longer, there is invariably a 'breakthrough' of virus in which virus that was at first neutralised appears to disassociate from the antibody.

ix) *Interpretation of the results:* The neutralisation index is the log titre difference between the titre of the virus in the negative serum and in the test serum. An index of ≥1.5 is positive. The test can be made more sensitive if serum from the same animal is examined before and after infection. Because the immunity to capripoxviruses is predominantly cell mediated, a negative result, particularly following vaccination, after which the antibody response may be low, does not imply that the animal from which the serum was taken is not protected.

Antibodies to capripoxvirus can be detected from 1 to 2 days after the onset of clinical signs. These remain detectable for about 7 months.

2.2. Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assays (ELISAs) for the detection of capripoxviral antibodies are widely used and are available in commercial kit form (Milovanovic *et al.,* 2019; Samojlovic *et al.,* 2019).

2.3. Indirect fluorescent antibody test

Capripoxvirus-infected tissue culture grown on cover-slips or tissue culture microscope slides can be used for the indirect fluorescent antibody test. Uninfected tissue culture control, and positive and negative control sera, should be included in the test. The infected and control cultures are fixed in acetone at –20°C for 10 minutes and stored at 4°C. Dilutions of test sera are made in PBS, starting at 1/20 or 1/40, and positive samples are identified using an anti-bovine gamma-globulin conjugated with fluorescein isothiocyanate. Antibody titres may exceed 1/1000 after infection. Sera may be screened at 1/50 and 1/500. Cross-reactions can occur with orf virus (contagious pustular dermatitis virus of sheep), bovine papular stomatitis virus and perhaps other poxviruses.

2.4. Western blot analysis

Western blotting of test sera against capripoxvirus-infected cell lysate provides a sensitive and specific system for the detection of antibody to capripoxvirus structural proteins, although the test is expensive and difficult to carry out.

Capripoxvirus-infected LT cells should be harvested when 90% CPE is observed, freeze–thawed three times, and the cellular debris pelleted using centrifugation. The supernatant should be decanted, and the proteins should be separated using SDS/PAGE (sodium dodecyl sulphate/polyacrylamide gel electrophoresis). A vertical discontinuous gel system, using a stacking gel made up of acrylamide (5%) in Tris (125 mM), pH 6.8, and SDS (0.1%), and a resolving gel made up of acrylamide (10–12.5%) in Tris (560 mM), pH 8.7, and SDS (0.1%), is recommended for use with a glycine running buffer containing Tris (250 mM), glycine (2 M), and SDS (0.1%). Samples of supernatant should be prepared by boiling for 5 minutes with an appropriate lysis buffer prior to loading. Alternatively, purified virus or recombinant antigens may replace tissue-culture-derived antigen.

Molecular weight markers should be run concurrently with the protein samples. The separated proteins in the SDS/PAGE gel should be transferred electrophoretically to a nitrocellulose membrane (NCM). After transfer, the NCM is rinsed thoroughly in PBS and blocked in 3% bovine serum albumin (BSA) in PBS, or 5% skimmed milk powder in PBS, on a rotating shaker at 4□C overnight. The NCM can then be separated into strips by employing a commercial apparatus to allow the concurrent testing of multiple serum samples, or may be cut into strips and each strip incubated separately thereafter. The NCM is washed thoroughly with five changes of PBS for 5 minutes on a rotating shaker, and then incubated at room temperature on the shaker for 1.5 hours, with the appropriate serum at a dilution of 1/50 in blocking buffer (3% BSA and 0.05% Tween 20 in PBS; or 5% milk powder and 0.05% Tween 20 in PBS). The membrane is again thoroughly washed and incubated (in blocking buffer) with anti-species immunoglobulin horseradish-peroxidase-conjugated immunoglobulins at a dilution determined using titration. After further incubation at room temperature for 1.5 hours, the membrane is washed and a solution of diaminobenzidine tetrahydrochloride (10 mg in 50 ml of 50 mM ~~mm~~–Tris/HCl, pH 7.5, and 20 µl of 30% [v/v] hydrogen peroxide) is added. Incubation is then undertaken for approximately 3–7 minutes at room temperature on a shaker with constant observation, and the reaction is stopped by washing the NCM in PBS before excessive background colour is seen. A positive and negative control serum should be used on each occasion.

Positive test samples and the positive control will produce a pattern consistent with reaction to proteins of molecular weights 67, 32, 26, 19 and 17 kDa – the major structural proteins of capripoxvirus – whereas negative serum samples will not react with all these proteins. Hyperimmune

serum prepared against parapoxvirus (bovine papular stomatitis or pseudocowpox virus) will react with some of the capripoxvirus proteins, but not the 32 kDa protein that is specific for capripoxvirus.

C. REQUIREMENTS FOR VACCINES

1. Background: rationale and intended use of the product

~~Live attenuated strains of capripoxvirus have been used as vaccines specifically for the control of LSD (Brenner *et al.*, 2006; Capstick & Coakley, 1961; Carn, 1993). Capripoxviruses are cross-reactive within the genus. Consequently, it is possible to protect cattle against LSD using strains of capripoxvirus derived from sheep or goats (Coakley & Capstick, 1961). However, it is recommended to carry out controlled trials, using the most susceptible breeds, prior to introducing a vaccine strain not usually used in cattle. The duration of protection provided by LSD vaccination is unknown.~~

~~Capripoxvirus vaccine strains can produce a large local reaction at the site of inoculation in *Bos taurus* breeds (Davies, 1991), which some stock owners find unacceptable. This has discouraged the use of vaccine, even though the consequences of an outbreak of LSD are invariably more severe. Risk–benefit of vaccination should be assessed following stakeholder discussion.~~

Vaccines are a key tool to control LSD. Different types of LSD vaccines have been developed and several are commercially available (Tuppurainen *et al.,* 2021).

Live attenuated vaccines (LAV) based on the Neethling LSDV strain (homologous LAV vaccines) have been shown to offer high levels of protection against LSD under experimental conditions (Haegeman *et al.,* 2021) and have been used successfully to control the disease in the field, through systematic vaccination of the entire country's cattle population for a number of consecutive years (Klement *et al.,* 2020). Homologous vaccines may induce fever, produce a local reaction at the site of inoculation, cause a temporary reduction in milk production and on rare occasions induce a 'Neethling' response (Ben-Gera *et al.,* 2015; Davies, 1991; Haegeman *et al.,* 2021). Such adverse effects, however, usually resolve within a few days and are largely outweighed by the overall benefits of vaccination with homologous vaccines. The duration of immunity induced by good quality live attenuated LSDV vaccines was shown to be at least 18 months (Haegeman *et al.,* 2023).

As capripoxviruses provide cross-reactive protection within the genus, heterologous LAVs comprising sheeppox virus or goatpox virus strains have also been tested and used to protect cattle against LSD. Sheeppox virus-based heterologous vaccines usually contain higher doses of virus than when administered to sheep. Although safe, their effectiveness in protecting cattle against LSD is inferior compared to homologous vaccines (Ben-Gera *et al*., 2015; Zhugunissov *et al*., 2020). Heterologous vaccines containing goatpox virus strains for use in cattle against LSD have been developed more recently. One such vaccine based on the Gorgan strain provided protection under experimental conditions comparable to homologous vaccines (Gari *et al*., 2015). On the other hand, a goat pox vaccine based on an attenuated Uttarkashi goatpox virus strain performed suboptimally under field conditions in India (Naveem *et al*., 2023), indicating that further research is warranted before asserting that all goatpox virus-based vaccines induce protection equal to homologous vaccines in cattle against LSD.

In addition, homologous inactivated vaccines against LSD have been developed and tested (Haegeman *et al*., 2023; Hamdi *et al*., 2020; Wolff *et al*., 2022). These vaccines are reported to be safe and efficacious. They however require a booster vaccination one month after primo-vaccination and then every 6 months thereafter, based on the fact that the duration of immunity is shorter than 1 year (Haegeman *et al*., 2023).

None of the commercial vaccines currently available has practical DIVA capacity. This problem may be resolved in the future by introducing new types of vaccines (e.g. vector-vaccines, subunit vaccines, mRNA vaccines) that are at various stages of development and evaluation.

2. Outline of production of LSD vaccines ~~and minimum requirements for conventional vaccines~~

~~General requirements set for the facilities used for the production of vaccines and for the documentation and record keeping throughout the whole manufacturing process are described in Chapter 1.1.8 *Principles of veterinary vaccine production*. The documentation should include standard operating procedures (SOP) for the method of manufacture and each step for the testing of cells and reagents used in the process, each batch and the final product.~~

The production of vaccines, including LSD vaccines, starts within research and development (R&D) facilities where vaccine candidates are produced and tested in preclinical studies to demonstrate the quality, safety and efficacy of the product.

Minimum requirements for different production stages of veterinary vaccines are available in different chapters of the *Terrestrial Manual.* These are intended to be used in combination with country-specific regulatory requirements for vaccine production and release. Here we outline the most important requirements for the production of live and inactivated LSD vaccines. Full requirements are available in Chapter 1.1.8 *Principles of veterinary vaccine production,* Chapter 2.3.3 *Minimum requirements for the organisation and management of a vaccine manufacturing facility* and Chapter 2.3.4 *Minimum requirements for the production and quality control of vaccine,* and other regulatory documentation.

2.1. Quality assurance

Facilities for manufacturing LSD vaccines should operate in line with the concepts of good laboratory practice (GLP) and good manufacturing practice (GMP) to produce high quality products. Quality risk management and quality control with adequate documentation management, as an integral part of the production process, have to be in place. In case some activities of the production process are outsourced, those should also be appropriately defined, recorded and controlled.

The vaccine production process (Outline of Production) should be documented in a series of standard operating procedures (SOPs), or other documents describing the manufacturing of each batch and the final product (including starting materials to be used, manufacturing steps, in-process controls and controls on the final product). Detailed requirements for documentation management in the process of vaccine production are available in Chapter 2.3.3.

A completed Outline of Production is to be enclosed in a vaccine candidate dossier and used for the evaluation of the production process and product by regulatory bodies.

2.2. Process validation

The dossier with the enclosed Outline of Production for the vaccine candidate has to be submitted for regulatory approval, so it can be assessed and authorised by the competent authority to ensure compliance with local regulatory requirements. Among others, data on quality, safety, and efficacy will be assessed. The procedures necessary to obtain these data are described in the subsequent sections.

National regulatory authorities might also require official control authority re-testing (check testing) of final products and batches in government laboratories or an independent batch quality control by a third party.

3. Requirements for LSD vaccine candidates and batch production

3.1. Requirements for starting materials

Live attenuated vaccines (LAV) and inactivated vaccines (IV) for LSD are produced using the system of limited and controlled passages of master seed and working seed virus and cell banks with a specified maximum. This approach aims to prevent possible and unwanted drift of properties of seed virus and cells that might arise from repeated passaging.

3.1.1. Characteristics of the seed virus

~~Each seed strain of capripoxvirus used for vaccine production must be accompanied by records clearly and accurately describing its origin, isolation and tissue culture or animal passage history. Preferably, the species and strain of capripoxvirus are characterised using PCR or DNA sequencing techniques.~~

~~A quantity of master seed vaccine virus should be prepared, frozen or desiccated and stored at low temperatures such as –80°C and used to produce a consistent working seed for regular vaccine production.~~

~~Each master seed strain must be non-transmissible, remain attenuated after further tissue culture passage, and provide complete protection against challenge with virulent field strains for a minimum of 1 year. It must produce a minimal clinical reaction in cattle when given via the recommended route.~~

~~The necessary safety and potency tests are described in Section C.2.2.4 *Final product batch tests*.~~

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

~~Each master seed must be tested to ensure its identity and shown to be free from adventitious viruses, in particular pestiviruses, such as border disease and bovine viral diarrhoea virus, and free from contamination with bacteria, fungi or mycoplasmas.~~

~~The general procedures for sterility or purity tests are described in Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*.~~

Master seed virus is a quantity of virus of uniform composition derived from an original isolate, passaged for a documented number of times and distributed into containers at one time and stored adequately to ensure stability (via freezing or lyophilisation). Selection of master seed viruses (MSVs) should ideally be based on their ease of growth in cell culture, virus yield, and in accordance with the regional epidemiological importance. Also, measures to minimise transmissible spongiform encephalopathies (TSE) contamination should be taken into account (see Section C.3.5.1 *Purity tests*).

For each seed strain selected for LSD vaccine production, the following information should be provided:

- Historical record: geographical origin, animal species from which the virus was recovered, isolation procedure, tissue culture or animal passage history
- Identity: species and strain identification using DNA sequencing
- Purity: the absence of bacteria, fungi, mycoplasma, and other viruses (see Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*)
- Safety (overdose, one/repeated dose tests, and reversion to virulence tests) (see Section C.3.3 *Vaccine safety*)
- Efficacy data, linked to a specified (protective) dose (see Section C.3.4 *Vaccine efficacy*)
- Stability

Each master seed strain selected for production of live attenuated LSD vaccines must remain attenuated after further passage in animals (see Section C.3.3. *Vaccine safety*), produce minimal clinical reaction when given via the recommended route, provide complete protection against challenge with virulent field strains, and is ideally not transmissible.

A quantity of master seed virus should be prepared and stored to be further used for the preparation of working seeds and production seeds. Working seed viruses may be expanded

in one or more (but, limited) cell culture passages from the master seed stock and used to produce vaccine batches. This approach and limitation of seed virus passaging will assist in maintaining uniformity and consistency in production.

3.1.2. Master cell stocks

The production process of LSD vaccines ideally employs an established master cell stock (MCS) system with defined lowest and highest cell passage to be used to grow the vaccine virus. Primary cells derived from normal tissues can be used in the production process, but the use of primary cells has an inherently higher risk of introducing extraneous agents compared with the use of established (well characterised) cell lines and should be avoided where alternative methods of producing effective vaccines exist. For each MCS, manufacturers should demonstrate:

- MCS identity
- genetic stability by subculturing from the lowest to the highest passage used for production
- stable MCS karyotype with a low level of polyploidy
- freedom from oncogenicity or tumorigenicity by using *in-vivo* studies using the highest cell passage that may be used for production
- purity of MCSs from extraneous bacteria, fungi, mycoplasma, and viruses
- implemented measures to lower TSE contamination risk (see Section C.3.5.1 *Purity tests*).

3.2. Method of vaccine manufacturing

~~The method of manufacture should be documented as the Outline of Production.~~

~~2.2.1. Procedure~~

3.2.1. LSD vaccine batch production

~~Vaccine batches are produced on an appropriate cell line such as MDBK.~~ As already mentioned in the first paragraphs of Section C, all steps undertaken in the production of vaccine batches should be described and documented in the Outline of Production. The production of LAV and IV against LSD starts with the inoculation of the required number of working vials of seed virus ~~is~~ reconstituted ~~with GMEM or other~~ in appropriate medium ~~and inoculated~~ onto a suitable primary or continuous cell line grown in suspension or monolayer~~. Cells should be harvested after 4–8 days when they exhibit 50–70% CPE for maximum~~ in the exponential growth phase. At the time highest viral ~~infectivity, or earlier if CPE is extensive and cells appear ready to detach. Techniques such as~~ loads are present, sonication or repeated freeze–thawing ~~are~~ is used to release the intracellular virus from the cytoplasm. The lysate may then be clarified using centrifugation to remove cellular debris ~~(for example by use of centrifugation at 600 ***g*** for 20 minutes~~, with retention of the supernatant~~)~~. ~~A second passage of the virus may be required to produce sufficient virus for a production batch.~~

An aliquot of the virus suspension is titrated to check the virus titre. For LAV, the virus-containing suspension is diluted to attain the dose at which the vaccine candidate will be evaluated or to at least the determined protective dose for approved vaccines and is then mixed with a suitable protectant ~~such as an equal volume of sterile, chilled 5% lactalbumin hydrolysate and 10% sucrose (dissolved in double-distilled water or appropriate balanced salt solution),~~ and transferred to individually ~~numbered~~ labelled bottles or bags for storage at low temperatures such as –80°C, or for freeze–drying. A written record of all the procedures followed must be kept for all vaccine batches.

~~2.2.2. Requirements for substrates and media~~

~~The specification and source of all ingredients used in the manufacturing procedure should be documented and the freedom of extraneous agents (bacteria, fungi, mycoplasma and viruses) should be tested. The detailed testing procedure is described in Chapter 1.1.9. The use of antibiotics must meet the requirements of the licensing authority.~~

~~2.2.3. In-process control~~

~~i) Cells~~

~~Records of the source of the master cell stocks should be maintained. The highest and lowest passage numbers of the cells that can be used for vaccine production must be indicated in the Outline of the Production. The use of a continuous cell line (such as MDBK, etc.) is strongly recommended, unless the virus strain only grows on primary cells. The key advantage of continuous over primary cell lines is that there is less risk of introduction of extraneous agents.~~

~~ii) Serum~~

~~Serum used in the growth or maintenance medium must be free from antibodies to capripoxvirus and free from contamination with pestivirus or other viruses, extraneous bacteria, mycoplasma or fungi.~~

~~iii) Medium~~

~~Media must be sterile before use.~~

~~iv) Virus~~

~~Seed virus and final vaccine must be titrated and pass the minimum release titre set by the manufacturer. For example, the minimum recommended field dose of the South African Neethling strain vaccines (Mathijs *et al.,* 2016) is $\log_{10}$ 3.5 $TCID_{50}$, although the minimum protective dose is $\log_{10}$ 2.0 $TCID_{50}$. Capripoxvirus is highly susceptible to inactivation by sunlight and allowance should be made for loss of activity in the field.~~

~~The recommended field dose of the Romanian sheep pox vaccine for cattle is $\log_{10}$ 2.5 sheep infective doses (SID_{50}), and the recommended dose for cattle of the RM65-adapted strain of Romanian sheep pox vaccine is $\log_{10}$ 3 $TCID_{50}$ (Coakley & Capstick, 1961).~~

3.2.2. Inactivation process for inactivated LSD vaccines

Unlike LAV, inactivated LSD vaccines contain inactivated antigens in combination with adjuvants to strengthen the induced immune response after administration. The vaccine evaluation process described below needs to show the amount of antigen necessary to elicit a protective immune response. Currently, literature data indicate that an inactivated vaccine originating from an LSDV virus stock with titre 10^4 cell culture infectious $dose_{50}$ ($CCID_{50}$)/ml before inactivation can be sufficient to induce an efficient immune response to prevent clinical disease, viremia and virus shedding after challenge of young cattle (Wolf *et al.,* 2022)

To monitor the inactivation process and the level of antigen inactivation, samples are taken at regular intervals during inactivation and titrated. Inactivation conditions and the length of initial and repeated exposure should be documented in detail since one or more factors during the process could influence the outcomes. The inactivation kinetics should reach a predefined target e.g. one remaining infectious unit per million doses (1×10^{-6} infectious units/dose) as suggested by APHIS (2013). The confirmatory testing of inactivation is performed on each vaccine lot and represents an important part of the inactivation process monitoring. In addition to all the procedures mentioned above, the inactivation procedure and tests demonstrating that antigen inactivation is complete and consistent must additionally be documented in the Outline of Production.

3.3. Vaccine safety

During the vaccine development process, vaccine safety must be evaluated in the target animal (target animal batch safety test –TABST) to demonstrate the safety of the dose intended for registration. The animals used in the safety testing should be representative (species, age and category [calves, heifers, bulls, cows.]) for all the animals for which the vaccine is intended. Vaccinated and control groups are appropriately acclimatised, housed and managed in line with animal welfare standards. Animal suffering has to be eliminated or reduced and euthanasia is recommended in moribund animals.

Essential parameters to be evaluated in safety studies are local and systemic reactions to vaccination, including local reactions at the site of administration, fever, effect on milk production, and induction of a 'Neethling' response. The effect of the vaccine on reproduction needs to be evaluated where applicable.

A part of the safety evaluation of LAV and IV can be performed during the efficacy trials (see Section C.3.4 *Vaccine efficacy*) by measuring local and systemic responses following vaccination and before challenge.

Guidelines for safety evaluation are provided by the European Medicine Agency (EMEA) in VICH GL44: TABST for LAV and IV (EMEA, 2009). Safety aspects of LAV and IV against LSD to be evaluated are:

3.3.1. Overdose test for LAV

Local and systemic responses should be measured following an overdose test whereby 10× the maximum vaccine titre is administered. If the maximum vaccine titre is not specified, 10× the minimum vaccine titre can be applied in multiple injection sites. Ideally, the 10× dose is dissolved in the 1× dose volume of the adjuvants or diluent. Generally, eight animals per group should be used (EMEA, 2009).

3.3.2. One dose and repeat dose test

This aims to test the safety of the vaccine dose applied in the vaccination regime intended for registration. LAV LSD vaccines require one dose per year, while inactivated LSD vaccines require a booster dose in addition to the primary dose. The minimal recommended interval between administrations is 14 days.

Generally, eight animals per group should be used unless otherwise justified (EMEA, 2009). For each target species, the most sensitive breed, age and sex proposed on the label should be used. Seronegative animals should be used. In cases where seronegative animals are not reasonably available, alternatives should be justified.

3.3.3. Reversion to virulence tests

Live attenuated vaccines inherently carry the risk of vaccine virus reverting to virulence when repeated passages in a host species could occur due to shedding and transmission from vaccinated animals to contact animals. LAV LSD vaccines should therefore be tested for non-reversion to virulence by means of passage studies. Vaccine virus (MSV, not the finished vaccine) is inoculated in a group of target animals of susceptible age via the natural route of infection or the route that is most likely to result in infection. The vaccine virus is subsequently recovered from tissues or excretions and is used directly to inoculate a further group of animals. After not less than four passages (see chapter 1.1.8), i.e. use of a total of five groups of animals, the re-isolate must be fully characterised, using the same procedures used to characterise the master seed virus.

3.3.4. Environmental consideration

This includes the evaluation of the ability of LAV LSD vaccines to be shed, to spread and to infect contact target and non-target animals, and to persist in the environment.

~~**2.2.4. Final product batch tests**~~

~~i) Sterility/purity~~

~~Vaccine samples must be tested for sterility/purity. *Tests for sterility and freedom from contamination of biological materials intended for veterinary use* may be found in Chapter 1.1.9.~~

~~ii) Safety and efficacy~~

~~The efficacy and safety studies should be demonstrated using statistically valid vaccination–challenge studies using seronegative young LSDV susceptible dairy cattle breeds. The group numbers recommended here can be varied if statistically justified. Fifteen cattle are placed in a high containment level large animal unit and serum samples are collected. Five randomly chosen vials of the freeze-dried vaccine are reconstituted in sterile PBS and pooled. Two cattle are inoculated with 10 times the recommended field dose of the vaccine, and eight cattle are inoculated with the recommended field dose. The remaining five cattle are unvaccinated control animals. The animals are clinically examined daily and rectal temperatures are recorded. On day 21 after vaccination, the animals are again serum sampled and challenged with a known virulent capripoxvirus strain. The challenge virus solution should also be tested free from extraneous viruses. The clinical response is recorded during the following 14 days. Animals in the unvaccinated control group should develop the typical clinical signs of LSD, whereas there should be no local or systemic reaction in the vaccinates other than a raised area in the skin at the site of vaccination, which should disappear after 4 days. Serum samples are again collected on day 30 after vaccination. The day 21 serum samples are examined for seroconversion to selected viral diseases that could have contaminated the vaccine, and the days 0 and 30 samples are compared to confirm the absence of antibody to pestivirus. Because of the variable response in cattle to LSD challenge, generalised disease may not be seen in all of the unvaccinated control animals, although there should be a large local reaction.~~

~~Once the efficacy of the particular strain being used for vaccine production has been determined in terms of minimum dose required to provide immunity, it is not necessary to repeat this on the final product of each batch, provided the titre of virus present has been ascertained.~~

~~iii) Batch potency~~

~~Potency tests in cattle must be undertaken for vaccine strains of capripoxvirus if the minimum immunising dose is not known. This is usually carried out by comparing the titre of a virulent challenge virus on the flanks of vaccinated and control animals. Following vaccination, the flanks of at least three animals and three controls are shaved of hair. Log_{10} dilutions of the challenge virus are prepared in sterile PBS and six dilutions are inoculated intradermally (0.1 ml per inoculum) along the length of the flank; four replicates of each dilution are inoculated down the flank. An oedematous swelling will develop at possibly all 24 inoculation sites on the control animals, although preferably there will be little or no reaction at the four sites of the most dilute inocula. The vaccinated animals may develop an initial hypersensitivity reaction at sites of inoculation within 24 hours, which should quickly subside. Small areas of necrosis may develop at the inoculation site of the most concentrated challenge virus. The titre of the challenge virus is calculated for the vaccinated and control animals; a difference in titre >log_{10} 2.5 is taken as evidence of protection.~~

3.4. Vaccine efficacy

Data enclosed in the vaccine candidate dossier should support the efficacy of the vaccine in each animal species for each vaccination regimen that is described in the product label recommendation. This includes studies regarding the onset of protection when claims for onset are made and for the duration of immunity. Efficacy studies should be conducted with the vaccine candidate that has been

produced at the highest passage level permitted for vaccine production as specified in the Outline of Production.

Efficacy (and safety) should be demonstrated in vaccination–challenge studies using representative (by species, age and category) seronegative healthy animals for which the vaccine is intended and which are tested negative for standard viral pathogens.

An example of a vaccination-challenge test set-up is outlined here. The group numbers mentioned can be varied if statistically justified. Thirteen animals are placed in a high containment large animal unit and are divided into two groups:

- single/repeated dose test group (*n*=8) – animals inoculated with the vaccine dose and route intended for registration (in case of an IV against LSD, a booster dose should follow primary vaccination after minimum 14 days).
- control group (*n*=5) – non-vaccinated animals

Throughout the *in-vivo* study, all animals are clinically examined and rectal temperatures recorded. Blood, serum and swab samples are regularly collected and subjected to laboratory testing. On day 21 after the vaccination with a LAV or after the booster vaccination for an IV, the animals in both groups are challenged with a known virulent LSDV strain. The challenge virus solution should be of known titre and tested free from extraneous viruses. Experience obtained from previous animal experiments indicates that a dose of challenge virus between $10^{4.0}$ and $10^{6.5}$ $TCID_{50}$ produces clinical disease in about half of the susceptible experimental cattle (Tuppurainen *et al.*, 2021).

The clinical response following challenge is recorded over a period of 14 days. No clinical signs should occur in the vaccinates, other than a local reaction at the site of inoculation. At least 1 animal in the unvaccinated control group should develop the typical clinical signs of LSD. Although a generalised disease with skin nodules may not be seen in all the unvaccinated control animals based on the knowledge that the outcome of a LSDV infection can range from inapparent to severe, at the very least a large local reaction is to be expected.

Clinical and laboratory results will enable assessment of the safety and efficacy of the LSD vaccine candidate and the induced immune responses. Serum samples collected at different time points during the trial can be examined to study seroconversion against selected viral diseases that could have contaminated the vaccine.

~~2.3. Requirements for regulatory approval~~

~~2.3.1. Safety requirements~~

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

~~The vaccine must be safe to use in all breeds of cattle for which it is intended, including young and pregnant animals. It must also be non-transmissible and remain attenuated after further tissue culture passage.~~

~~Safety tests should be carried out on the final product of each batch as described in Section C.2.2.4.~~

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

~~The selected final vaccine should not revert to virulence during further passages in target animals.~~

~~iii) Environmental consideration~~

~~Attenuated vaccine should not be able to perpetuate autonomously in a cattle population. Strains of LSDV are not a hazard to human health.~~

~~2.3.2. Efficacy requirements~~

~~i) For animal production~~

~~The efficacy of the vaccine must be demonstrated in statistically valid vaccination challenge experiments under laboratory conditions. The group numbers recommended here can be varied if statistically justified. Fifteen cattle are placed in a high containment level large animal unit and serum samples are collected. Five randomly chosen vials of the freeze-dried vaccine are reconstituted in sterile PBS and pooled. Two cattle are inoculated with 10 times the field dose of the vaccine, eight cattle are inoculated with the recommended field dose. The remaining five cattle are unvaccinated control animals. The animals are clinically examined daily and rectal temperatures are recorded. On day 21 after vaccination, the animals are again serum sampled and challenged with a known virulent capripoxvirus strain using intravenous and intradermal inoculation (the challenge virus solution should also be tested and shown to be free from extraneous viruses). The clinical response is recorded during the following 14 days. Animals in the unvaccinated control group should develop the typical clinical signs of LSD, whereas there should be no local or systemic reaction in the vaccinates other than a raised area in the skin at the site of vaccination which should disappear after 4 days. Serum samples are again collected on day 30 after vaccination. The day 21 serum samples are examined for seroconversion to selected viral diseases that could have contaminated the vaccine, and the days 0 and 30 samples are compared to confirm the absence of antibody to pestivirus. Because of the variable response in cattle to challenge with LSDV, generalised disease may not be seen in all of the unvaccinated control animals, although there should be a large local reaction.~~

~~Once the potency of the particular strain being used for vaccine production has been determined in terms of minimum dose required to provide immunity, it is not necessary to repeat this on the final product of each batch, provided the titre of virus present has been ascertained.~~

~~ii) For control and eradication~~

~~Vaccination is the only effective way to control LSD outbreaks in endemic countries and recent experiences of the disease in Eastern Europe and the Balkans suggests this is also true for outbreaks in non-endemic countries. Unfortunately, currently no marker vaccines allowing a DIVA strategy are available, although to a limited extent PCR can be used for certain vaccines.~~

~~The duration of immunity produced by LSDV vaccine strains is currently unknown.~~

~~2.3.3. Stability~~

~~All vaccines are initially given a shelf life of 24 months before expiry. Real-time stability studies are then conducted to confirm the appropriateness of the expiry date. Multiple batches of the vaccine should be re-titrated periodically throughout the shelf-life period to determine the vaccine stability.~~

~~Properly freeze-dried preparations of LSDV vaccine, particularly those that include a protectant, such as sucrose and lactalbumin hydrolysate, are stable for over 25 years when stored at –20°C and for 2–4 years when stored at 4°C. There is evidence that they are stable at higher temperatures, but no long-term controlled experiments have been reported. No preservatives other than a protectant, such as sucrose and lactalbumin hydrolysate, are required for the freeze-dried preparation.~~

3.5. Batch/serial tests before release for distribution

Quality tests on MSV and safety and efficacy tests on vaccine candidates are performed during the evaluation process for new LSD vaccines. Once vaccines are approved to be used in the field, it remains important to verify the quality of each vaccine batch produced. An independent batch quality control assessment may be warranted or requested by national or international regulatory authorities.

3.5.1. Purity test

Purity is defined by the absence of different contaminants (bacteria, fungi, mycoplasma, and other viruses; see full details in chapter 1.1.9) in the vaccine and its associated diluent/adjuvants. Virus isolation and bacterial culture tests can be used to show freedom from live competent replicating microorganisms, but molecular methods are more rapid and sensitive, but positives can be caused by genome fragments and incompetent replicating microorganisms.

Besides the contaminants mentioned above, manufacturers should demonstrate implemented measures to minimise the risk of TSE contamination in ingredients of animal origin such as:

- all ingredients of animal origin in production facilities are from countries recognised as having the lowest possible risk of bovine spongiform encephalopathy
- tissues or other substances used are themselves recognised as being of low or nil risk of containing TSE agents

3.5.2. Identity tests

In addition to identity tests performed on the MSV, the identity tests on final batches aim to demonstrate the presence of only the selected capripoxvirus species and strain in the vaccine as indicated in the Outline of Production and the absence of other strains or members of the genus and any other viral contaminant that might arise during the production process. Identity testing could be assured by using appropriate tests (e.g. PCRs, sanger sequencing, NGS).

3.5.3. Potency tests

Standard requirements for potency tests can be found in CFR Title 9 part 113, in the European Pharmacopoeia, and in this Terrestrial Manual.

3.5.3.1. Live vaccines

The potency of LAV against LSD can be measured by means of virus titration. The virus titre must, as a rule, be sufficiently greater than that shown to be protective in the efficacy test for the vaccine candidate. This will ensure that at any time prior to the expiry date, the titre will be at least equal to the evaluated protective titre. The titres of currently available commercial homologous LSD vaccines range between 10^3 and 10^4 infectious units/dose (Tuppurainen *et al*., 2021).

3.5.3.2. Inactivated LSD vaccines

For inactivated LSD vaccines, potency tests are performed using vaccination–challenge efficacy studies in animal hosts (see Section C.3.4. *Vaccine efficacy*).

3.5.4. Safety/efficacy

Safety and efficacy testing is undertaken during the evaluation process of the vaccine candidate, and also needs to be performed on a number of vaccine batches until robust data are generated in line with international and national regulations. Afterwards, when using a seed lot system in combination with strict implementation of GMP standards and depending on local regulations, TABST could be waived as described in VICH50 and VICH55, providing the titer has been ascertained using potency testing. Batches or serials are considered satisfactory if local and systemic reactions to vaccination are in line with those described in the dossier of the vaccine candidate and product literature.

3.5.4.1. Field safety/efficacy tests

Field testing of two or more batches should be performed on all animal categories for which the product is indicated before release of the product for general use (see chapter 1.1.8). The aim of these studies is to demonstrate the safety and efficacy of the product under normal field conditions of animal care and use in different geographical locations where different factors may influence product performance. A protocol for safety/efficacy testing in the field has to be developed with defined observation and recording procedures. However, it is generally more difficult to obtain statistically significant data to demonstrate efficacy under field conditions. Even when properly designed, field efficacy studies may be inconclusive due to uncontrollable outside influences.

3.5.4.2. Duration of Immunity

The duration of immunity (DOI) following vaccination should be demonstrated via challenge or the use of a validated serology test. Efficacy testing at the end of the claimed period of protection should be conducted in each species for which the vaccine is indicated or the manufacturer should indicate that the DOI for that species is not known. Likewise, the manufacturer should demonstrate the effectiveness of the recommended booster regime in line with these guidelines, usually by measuring the magnitude and kinetics of the serological response observed.

~~3. Vaccines based on biotechnology~~

~~A new generation of capripox vaccines is being developed that uses the LSDV as a vector for the expression and delivery of immuno-protective proteins of other ruminant pathogens with the potential for providing dual protection (Boshra *et al.*, 2013; Wallace & Viljoen, 2005), as well as targeting putative immunomodulatory genes for inducing improved immune responses (Kara *et al.*, 2018).~~

4. Post-market studies

4.1. Stability

Stability testing shall be carried out as specified in Annex II of Regulation (EU) 2019/6 and in the Ph. Eur. 0062: Vaccines for veterinary use, on not fewer than three representative batches providing this mimics the full-scale production described in the application. At the end of shelf-life, sterility has to be re-evaluated using sterility testing or by showing container closure integrity. Multiple batches of the vaccine should be re-titrated periodically throughout the shelf-life period to determine the vaccine stability.

4.2. Post-marketing surveillance

After release of a vaccine, its performance under field conditions should continue to be monitored by competent authorities and by the manufacturer itself. Not all listed adverse effects may show up in the clinical trials performed to assess safety and efficacy of the vaccine candidate due to the limited number of animals used. Post-marketing surveillance studies can also provide information on vaccine efficacy when used in normal practice and husbandry conditions, on duration of induced immunity, on ecotoxicity, etc.

First, a reliable reporting system should be in place to collect consumer complaints and notifications of adverse reactions. Secondly, post-marketing surveillance should be established to investigate whether the reported observations are related to the use of the product and to identify, at the earliest stage, any serious problem that may be encountered from its use and that may affect its future uptake. Vaccinovigilance should be an on-going and integral part of all regulatory programmes for LSD vaccines, especially for live vaccines.

REFERENCES

AGIANNIOTAKI E.I., CHAINTOUTIS S.C., HAEGEMAN A., TASIOUDI K.E., DE LEEUW I., KATSOULOS P.D., SACHPATZIDIS A., DE CLERCQ K., ALEXANDROPOULOS T., POLIZOPOULOU Z.S., CHONDROKOUKI E.D. & DOVAS C.I. (2017). Development and validation of a TaqMan probe-based real-time PCR method for the differentiation of wild type lumpy skin disease virus from vaccine virus strains. *J. Virol. Methods*, **249**, 48–57.

BABIUK S., BOWDEN T.R., PARKYN G., DALMAN B., MANNING L., NEUFELD J., EMBURY-HYATT C., COPPS J. & BOYLE D.B. (2008). Quantification of lumpy skin disease virus following experimental infection in cattle. *Transbound. Emerg. Dis.*, **55**, 299–307.

BABIUK S., PARKYN G., COPPS J., LARENCE J.E., SABARA M.I., BOWDEN T.R., BOYLE D.B. & KITCHING R.P. (2007). Evaluation of an ovine testis cell line (OA3.Ts) for propagation of capripoxvirus isolates and development of an immunostaining technique for viral plaque visualization. *J. Vet. Diagn.Invest.*, **19**, 486–491.

BALINSKY C.A, DELHON G, SMOLIGA G, PRARAT M, FRENCH R.A, GEARY S.J, ROCK D.L & RODRIGUEZ L.L. (2008). Rapid preclinical detection of sheep pox virus by a real-time PCR assay. *J. Clin. Microbiol.*, **46**, 438–442.

BEN-GERA J., KLEMENT E., KHINICH E, STRAM Y. & SHPIGEL N. (2015). Comparison of the efficacy of Neethling lumpy skin disease virus and x10RM65 sheep-pox live attenuated vaccines for the prevention of lumpy skin disease – The results of a randomized controlled field study. *Vaccine*, **33**, 4837–4842.

BISWAS S., NOYCE R.S., BABIUK L.A., LUNG O., BULACH D. M., BOWDEN T.R., BOYLE D. B., BABIUK S. & EVANS D.H. (2020). Extended sequencing of vaccine and wild-type capripoxvirus isolates provides insights into genes modulating virulence and host range. *Transbound. Emerg. Dis.*, **67**, 80–97.

~~BOSHRA H., TRUONG T., NFON C., GERDTS V., TIKOO S., BABIUK L.A., KARA P., MATHER A., WALLACE D. & BABIUK S. (2013). Capripoxvirus-vectored vaccines against livestock diseases in Africa. *Antiviral Res.*, **98**, 217–227.~~

BOWDEN, T.R, BABIUK S.L, PARKYN G.R., COPPS J.S. & BOYLE D.B. (2008). Capripoxvirus tissue tropism and shedding: A quantitative study in experimentally infected sheep and goats. *Virology*, **371**, 380–393.

BRENNER J., HAIMOVITZ M., ORON E., STRAM Y., FRIDGUT O., BUMBAROV V., KUZNETZOVA L., OVED Z., WASERMAN A., GARAZZI S., PERL S., LAHAV D., EDERY N. & YADIN H. (2006). Lumpy skin sease (LSD) in a large dairy herd in Israel. *Isr. J. Vet. Med.*, **61**, 73–77.

BURDIN M.L. (1959). The use of histopathological examination of skin material for the diagnosis of lumpy skin disease in Kenya. *Bull. Epizoot. Dis. Afr.*, **7**, 27–36.

~~BYADOVSKAYA O., PESTOVA Y., KONONOV A., SHUMILOVA I., KONONOVA S., NESTEROV A., BABIUK S. & SPRYGIN A. (2021). Performance of the currently available DIVA real-time PCR assays in classical and recombinant lumpy skin disease viruses. Transbound. Emerg. Dis., **68**, 3020-3024. doi: 10.1111/tbed.13942. Epub 2021 May 13. PMID: 33253485~~

~~CAPSTICK P.B. & COAKLEY W. (1961). Protection of cattle against lumpy skin disease. Trials with a vaccine against Neethling type infection. *Res. Vet. Sci.*, **2**, 362–368~~

~~CARN V.M. (1993). Control of capripoxvirus infections. *Vaccine*, **11**, 1275–1279.~~

CARN V.M. & KITCHING, R.P. (1995). The clinical response of cattle following infection with lumpy skin disease (Neethling) virus. *Arch. Virol.*, **140**, 503–513.

~~COAKLEY W. & CAPSTICK P.B. (1961). Protection of cattle against lumpy skin disease. Factors affecting small scale production of tissue culture propagated virus vaccine. *Res. Vet. Sci.*, **2**, 369–371.~~

COETZER J.A.W. (2004). Lumpy skin disease. *In:* Infectious Diseases of Livestock, Second Edition Coetzer J.A.W. & Justin R.C., eds. Oxford University Press, Cape Town, South Africa, 1268–1276.

953 DAO T.D., TRAN L.H., NGUYEN H.D., HOANG T.T., NGUYEN G.H., TRAN K.V.D., NGUYEN H.X., VAN DONG H., BUI
954 A.N. & BUI V.N. (2022). Characterization of Lumpy skin disease virus isolated from a giraffe in Vietnam.
955 *Transbound. Emerg. Dis.*, **69**(5):e3268-e3272. doi: 10.1111/tbed.14583. Epub 2022 May 10. PMID: 35502589

956 DAS A., BABIUK S. & MCINTOSH M.T. (2012). Development of a loop-mediated isothermal amplification assay for
957 rapid detection of capripoxviruses. *J. Clin. Microbiol.,* **50**, 1613–1620.

958 DAVIES F.G. (1991). Lumpy Skin Disease, a Capripox Virus Infection of Cattle in Africa. FAO, Rome, Italy.

959 DAVIES F.G., KRAUSS H., LUND L.J. & TAYLOR M. (1971). The laboratory diagnosis of lumpy skin disease. *Res.*
960 *Vet. Sci.*, **12**, 123–127.

961 FAY P.C., COOK C.G., WIJESIRIWARDANA N., TORE G., COMTET L., CARPENTIER A., SHIH B., FREIMANIS G., HAGA
962 I.R. & BEARD P.M. (2020). Madin-Darby bovine kidney (MDBK) cells are a suitable cell line for the propagation
963 and study of the bovine poxvirus lumpy skin disease virus. *J. Virol. Methods*, **285**, 113943. doi:
964 10.1016/j.jviromet.2020.113943.

965 FLANNERY J., SHIH B., HAGA I.R., ASHBY M., CORLA A., KING S., FREIMANIS G., POLO N., TSE A.C., BRACKMAN C.J.,
966 CHAN J., PUN P., FERGUSON A.D., LAW A., LYCETT S., BATTEN C. & BEARD P.M. (2021). A novel strain of lumpy
967 skin disease virus causes clinical disease in cattle in Hong Kong. *Transbound. Emerg. Dis.*, doi:
968 10.1111/tbed.14304

969 GARI G., ABIE G., GIZAW D., WUBETE A., KIDANE M., ASGEDOM H., BAYISSA B., AYELET G., OURA C.A.L., ROGER
970 F. & TUPPURAINEN E.S.M. (2015). Evaluation of the safety, immunogenicity and efficacy of three capripoxvirus
971 vaccine strains against lumpy skin disease virus. *Vaccine*, **33**, 3256–3261. doi: 10.1016/j.vaccine.2015.01.035.

972 HAIG D. (1957). Lumpy skin disease. *Bull. Epizoot. Dis. Afr.*, **5**, 421–430.

973 HEDGER R.S. & HAMBLIN C. (1983). Neutralising antibodies to lumpy skin disease virus in African wildlife. *Comp.*
974 *Immunol. Microbiol. Infect. Dis.*, **6**, 209–213. doi: 10.1016/0147-9571(83)90012-7. PMID: 6627909 DOI:
975 10.1016/0147-9571(83)90012-7

976 HAEGEMAN A., DE LEEUW I., MOSTIN L., VAN CAMPE W., AERTS L., VENTER E., TUPPURAINEN E., SAEGERMAN C. &
977 K. DE CLERCQ (2021). Comparative Evaluation of Lumpy Skin Disease Virus-Based Live Attenuated Vaccines.
978 *Vaccines*, **9**, 473. https://doi.org/10.3390/vaccines9050473

979 HAEGEMAN A., DE LEEUW I., MOSTIN L., VAN CAMPE W., PHILIPS W., ELHARRAK M., DE REGGE N. & DE CLERCQ K.
980 (2023). Duration of immunity induced after vaccination of cattle with a live attenuated or inactivated Lumpy
981 skin disease virus vaccine. *Microorganisms*, **11**, 210. https://doi.org/10.3390/microorganisms11010210

982 HAMDI J., BOUMART Z., DAOUAM S., EL ARKAM A., BAMOUH Z., JAZOULI M., OMARI TADLAOUI K., FASSI FIHRI O.,
983 GAVRILOV B. & EL HARRAK M. (2020). Development and Evaluation of an Inactivated Lumpy Skin Disease
984 Vaccine for Cattle. *Vet. Microbiol.*, **245**, 108689. doi: 10.1016/j.vetmic.2020.108689.

985 IRELAND D.C. & BINEPAL Y.S. (1998). Improved detection of capripoxvirus in biopsy samples by PCR. *J. Virol.*
986 *Methods*, **74**, 1–7.

987 IRONS P.C., TUPPURAINEN E.S.M. & VENTER E.H. (2005). Excretion of lumpy skin disease virus in bull semen.
988 *Theriogenology*, **63**, 1290–1297.

989 KARA P.D., AFONSO C.L., WALLACE D.B., KUTISH G.F., ABOLNIK C., LU Z., VREEDE F.T., TALJAARD L.C., ZSAK A.,
990 VILJOEN G.J. & ROCK D.L. (2003). Comparative sequence analysis of the South African vaccine strain and two
991 virulent field isolates of Lumpy skin disease virus. *Arch. Virol.*, **148**, 1335–1356.

992 ~~KARA P.D., MATHER A.S., PRETORIUS A., CHETTY T., BABIUK S. & WALLACE D.B. (2018). Characterisation of~~
993 ~~putative immunomodulatory gene knockouts of lumpy skin disease virus in cattle towards an improved vaccine.~~
994 ~~*Vaccine*, **36**, 4708-4715. doi: 10.1016/j.vaccine.2018.06.017.~~

KITCHING R.P. & SMALE C. (1986). Comparison of the external dimensions of capripoxvirus isolates. *Res. Vet. Sci.*, **41**, 425–427.

KLEMENT E., BROGLIA A., ANTONIOU S.-E., TSIAMADIS V., E. PLEVRAKI, PETROVIĆ T., POLAČEK V., DEBELJAK Z., MITEVA A., ALEXANDROV T., MAROJEVIC D., PITE L., KONDRATENKO V., ATANASOV Z., GUBBINS S., STEGEMAN A. & CORTIÑAS ABRAHANTES J. (2020). Neethling vaccine proved highly effective in controlling lumpy skin disease epidemics in the Balkans. *Prev. Vet. Med.*, **181**, 104595 https://doi.org/10.1016/j.prevetmed.2018.12.001

KUMAR R., GODARA B., CHANDER Y., KACHHAWA J.P., DEDAR R.K., VERMA A., RIYESH T., PAL Y., BARUA S., TRIPATHI B.N. & KUMAR N. (2023). Evidence of lumpy skin disease virus infection in camels. *Acta Trop.*, **242**, 106922. doi: 10.1016/j.actatropica.2023.106922. Epub 2023 Apr 7. PMID: 37031926

LAMIEN C.E., LELENTA M., GOGER W., SILBER R., TUPPURAINEN E., MATIJEVIC M., LUCKINS A.G. & DIALLO A. (2011). Real time PCR method for simultaneous detection, quantitation and differentiation of capripoxviruses *J. Virol. Methods*, **171**, 134–140.

~~MATHIJS E., VANDENBUSSCHE F., HAEGEMAN A., KING A., NTHANGENI B., POTGIETER C., MAARTENS L., VAN BORM S. &DE CLERCQ K. (2016). Complete Genome Sequences of the Neethling-Like Lumpy Skin Disease Virus Strains Obtained Directly from Three Commercial Live Attenuated Vaccines. *Genome Announc.*, **4** (6). pii: e01255-16. doi: 10.1128/genomeA.01255-16.~~

MILOVANOVIC M., DIETZE K., MILICEVIC V., RADOJICIC S., VALCIC M., MORITZ T. & HOFFMANN B. (2019). Humoral immune response to repeated lumpy skin disease virus vaccination and performance of serological tests. *BMC Vet. Res.*, **15**, 80. doi: 10.1186/s12917-019-1831-y.

MURRAY L., EDWARDS L., TUPPURAINEN E.S., BACHANEK-BANKOWSKA K., OURA C.A., MIOULET V. & KING D.P. (2013). Detection of capripoxvirus DNA using a novel loop-mediated isothermal amplification assay. *BMC Vet. Res.*, **9**, 90.

OMOGA D.C.A., MACHARIA M., MAGIRI E., KINYUA J., KASIITI J. & HOLTON T. (2016). Molecular based detection, validation of a LAMP assay and phylogenetic analysis of capripoxvirus in Kenya. *J. Adv. Biol. Biotech.*, **7**, 1–12.

PESTOVA Y., BYADOVSKAYA O., KONONOV A. & SPRYGIN A. (2018). A real time high-resolution melting PCR assay for detection and differentiation among sheep pox virus, goat pox virus, field and vaccine strains of lumpy skin disease virus. *Mol. Cell. Probes*, **41**, 57–60.

PORCO A., CHEA S., SOURS S., NOU V., GROENENBERG M., AGGER C., TUM S., CHHUON V., SORN S., HONG C., DAVIS B., DAVIS S., KEN S., OLSON S.H. & FINE A.E. (2023). Case report: Lumpy skin disease in an endangered wild banteng (Bos javanicus) and initiation of a vaccination campaign in domestic livestock in Cambodia. *Front. Vet. Sci.*, **10**:1228505. doi: 10.3389/fvets.2023.1228505

PROZESKY L. & BARNARD B.J.H. (1982). A study of the pathology of lumpy skin disease in cattle. *Onderstepoort J. Vet. Res.*, **49**, 167–175.

ROUBY S. & ABOULSOUDB E. (2016). Evidence of intrauterine transmission of lumpy skin disease virus. *Vet. J.*, **209**, 193–195.

SAMOJLOVIC M., POLACEK V., GURJANOV V., LUPULOVIC D., LAZIC G., PETROVIĆ T., LAZIC S. (2019). Detection of antibodies against lumpy skin disease virus by virus neutralization test and ELISA methods. *Acta Vet.*, **69**, 47–60.

SPRYGIN A., BABIN Y., PESTOVA Y., KONONOVA S., WALLACE D.B., VAN SCHALKWYK A., BYADOVSKAYA O., DIEV V., LOZOVOY D. & KONONOV A. (2018). Analysis and insights into recombination signals in lumpy skin disease virus recovered in the field. *PLoS One*, **13**, e0207480.

SPRYGIN A., VAN SCHALKWYK A., SHUMILOVA I., NESTEROV A., KONONOVA S., PRUTNIKOV P., BYADOVSKAYA O. & KONONOV A. (2020). Full-length genome characterization of a novel recombinant vaccine-like lumpy skin disease virus strain detected during the climatic winter in Russia, 2019. *Arch. Virol.*, **165**, 2675–2677.

TUPPURAINEN E., DIETZE K., WOLFF J., BERGMANN H., BELTRAN-ALCRUDO D., FAHRION A., LAMIEN EULOGE C., BUSCH F., SAUTER-LOUIS C., CONRATHS F.J., DE CLERCQ K., HOFFMANN B. & SASCHA K. (2021). Review: Vaccines and Vaccination against Lumpy Skin Disease. *Vaccines* (Basel). *9*, 1136. doi: 10.3390/vaccines9101136

TUPPURAINEN E.S.M., VENTER E.H. & COETZER J.A.W. (2005). The detection of lumpy skin disease virus in samples of experimentally infected cattle using different diagnostic techniques. *Onderstepoort J. Vet. Res.,* **72**, 153–164.

TUPPURAINEN E.S., VENTER E.H., COETZER J.A. & BELL-SAKYI L. (2015). Lumpy skin disease: attempted propagation in tick cell lines and presence of viral DNA in field ticks collected from naturally-infected cattle. *Ticks Tick Borne Dis*., **6**, 134–140.

VAN ROOYEN P.J., KOMM N.A.L., WEISS K.E. & ALEXANDER R.A. (1959). A preliminary note on the adaptation of a strain of lumpy skin disease virus to propagation in embryonated eggs. *Bull. Epizoot. Dis. Afr*., **7**, 79–85.

VAN SCHALKWYK A., BYADOVSKAYA O., SHUMILOVA I., WALLACE D.B. & SPRYGIN A. (2021). Estimating evolutionary changes between highly passaged and original parental lumpy skin disease virus strains. *Transbound. Emerg. Dis*., doi: 10.1111/tbed.14326.

VAN SCHALKWYK A., KARA P., EBERSOHN K., MATHER A., ANNANDALE C.H., VENTER E.H. & WALLACE D.B. (2020). Potential link of single nucleotide polymorphisms to virulence of vaccine-associated field strains of lumpy skin disease virus in South Africa. *Transbound. Emerg. Dis*., **67**, 2946–2960.

VIDANOVIC D., SEKLER M., PETROVIC T., DEBELJAK Z., VASKOVIC N., MATOVIC K. & HOFFMAN B. (2016). Real time PCR assay for the specific detection of field Balkan strain of lumpy skin disease virus. *Acta Vet. Brno*, **66**, 444–454.

WANG Y., ZHAO L., YANG J., SHI M., NIE F., LIU S., WANG Z., HUANG D., WU H., LI D., LIN H. & LI Y. (2021). Analysis of vaccine-like lumpy skin disease virus from flies near the western border of China. *Transbound. Emerg. Dis*., doi: 10.1111/tbed.14159

~~WALLACE D.B. & VILJOEN G.J. (2005). Immune responses to recombinants of the South African vaccine strain of lumpy skin disease virus generated by using thymidine kinase gene insertion. *Vaccine*, **23**, 3061–3067.~~

WEISS K.E. (1968). Lumpy skin disease. *Virol. Monogr*., **3**, 111–131.

WOLFF J., BEER M. & HOFFMANN B. (2022). High Efficiency of Low Dose Preparations of an Inactivated Lumpy Skin Disease Virus Vaccine Candidate. *Vaccines* (Basel), **10**, 1029. doi: 10.3390/vaccines10071029.

ZHUGUNISSOV K., BULATOV Y., ORYNBAYEV M., KUTUMBETOV L., ABDURAIMOV Y., SHAYAKHMETOV Y., TARANOV D., AMANOVA Z., MAMBETALIYEV M., ABSATOVA Z., AZANBEKOVA M., KHAIRULLIN B., ZAKARYA K. & TUPPURAINEN E. (2020). Goatpox virus (G20-LKV) vaccine strain elicits a protective response in cattle against lumpy skin disease at challenge with lumpy skin disease virulent field strain in a comparative study. *Vet Microbiol*., **245**, 108695. doi: 10.1016/j.vetmic.2020.108695.

*
* *

NB: There are WOAH Reference Laboratories for lumpy skin disease (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for lumpy skin disease

NB: FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2021.

Annex 13. Chapter 3.6.9. 'Equine rhinopneumonitis (infection with Varicellovirus equidalpha1)'

CHAPTER 3.6.9.

EQUINE RHINOPNEUMONITIS (INFECTION WITH VARICELLOVIRUS EQUIDALPHA1 ~~EQUID HERPESVIRUS-1 AND -4~~)

SUMMARY

Equine rhinopneumonitis (ER) is a collective term for any one of several contagious, clinical disease entities of equids that may occur as a result of infection by either of two closely related herpesviruses, formally known as equid ~~alpha~~herpesvirus-1 and -4 (EHV-1 and EHV-4). Infection with EHV-1 is listed by WOAH and is therefore the focus of this chapter. The classification of the virus has been reviewed and EHV-1 is now known as Varicellovirus equidalpha1. For the purposes of the chapter, the acronym EHV-1 will continue to be used. EHV-1 is ~~and EHV-4 are~~ endemic in most domestic equine populations worldwide.

Primary infection by ~~either~~ EHV-1 ~~or EHV-4~~ is characterised by upper respiratory tract disease of varying severity that is related to the age and immunological status of the infected animal. Following viraemia EHV-1 may also cause~~s~~ the more serious complications of abortion, perinatal foal death, or paralytic neurological disease (equine herpesvirus myeloencephalopathy). ~~EHV-4 has been associated with sporadic cases of abortion, but rarely multiple abortions and not the large outbreaks associated with EHV-1.~~ Like other herpesviruses, EHV-1 ~~and 4~~ induces long-lasting latent infections and can be reactivated following stress ~~or pregnancy~~. Furthermore, most horses are likely to be re-infected multiple times during their lifetime, often ~~mildly or~~ subclinically. ~~Detection of viral DNA or anti-EHV antibodies should therefore be interpreted with care.~~

***Identification of the agent:** The standard method of identification of EHV-1 ~~and EHV-4~~ from appropriate clinical or necropsy material is by polymerase chain reaction (PCR)~~, followed by laboratory isolation of the virus in cell culture~~.*

Positive identification of viral isolates as EHV-1 ~~or EHV-4~~ can be achieved by type-specific PCR or sequencing. Viruses can be isolated in ~~equine~~ cell culture from the following sample types: nasal or nasopharyngeal swab extracts taken from horses ~~during the febrile stage of~~ with acute respiratory tract infection, ~~from~~ the placenta, ~~from and~~ liver, lung, spleen, adrenal glands or thymus of aborted fetuses and early foal deaths, and from the leukocyte fraction of the blood of animals ~~with acute~~ during the febrile stage ~~of EHV-1 infection~~. ~~Unlike EHV-4, EHV-1 will also grow in various non-equine cell types such as the RK-13 cell line and this property can be used to distinguish between the two viruses.~~

A rapid presumptive diagnosis of abortion induced by EHV-1 ~~or (infrequently) EHV-4~~ can be achieved by direct immunofluorescent detection of viral antigen in cryostat sections of placenta and tissues from aborted fetuses, using a conjugated polyclonal antiserum.

Post-mortem demonstration of histopathological lesions of EHV-1 in placenta and tissues from aborted fetuses, cases of perinatal foal death, or in the central nervous system of neurologically affected animals complements other diagnostic techniques ~~the laboratory diagnosis~~.

Serological tests: *As most horses possess some level of antibody to EHV-1/4, the demonstration of specific antibody in the serum collected from a single blood sample is ~~therefore~~ not confirmation of ~~a positive diagnosis of~~ recent infection. Paired~~,~~ (acute and convalescent) sera from animals suspected of being infected with EHV-1 or EHV-4 should be tested for a four-fold or greater rise in virus-specific antibody titre by either virus neutralisation (VN) or complement fixation (CF) tests. Neither of these assays is type-specific but both have proven useful for diagnostic purposes especially since the CF antibody response to recent infection is relatively short-lived. Limited use has also been made of a type-specific enzyme-linked immunosorbent assay ~~(Crabb et al., 1995; Hartley et al., 2005)~~.*

Requirements for vaccines: *Both live attenuated and inactivated viral vaccines are available for use in assisting in the control of EHV-1~~/~~4. Vaccination is helpful in reducing the severity of respiratory infection in young horses and the incidence of abortion in mares; however current vaccines are not licenced to protect against neurological disease. Vaccination should not be considered a substitute for sound management practices known to reduce the risk of infection. Revaccination at frequent intervals is recommended in the case of each of the products, as the duration of vaccine-induced immunity is relatively short.*

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

A. INTRODUCTION

Equine rhinopneumonitis (ER) is a historically derived term that describes a constellation of several disease entities of horses that may include respiratory disease, abortion, neonatal foal pneumonitis, or myeloencephalopathy (~~Allen & Bryans, 1986;~~ Allen *et al*., 1999~~; Bryans & Allen, 1988; Crabb & Studdert, 1995~~). The disease ~~has been~~ is recognised ~~for over 60 years~~ as a threat to the international horse industry, and is caused by either of two members of the *Herpesviridae* family, formerly known as equid alphaherpesvirus-1 and -4 (EHV-1 and EHV-4). The viruses are now classified as Varicellovirus equidalpha1 and Varicellovirus equidalpha4. For the purposes of the chapter, the acronyms EHV-1 and EHV-4 will continue to be used. EHV-1 and EHV-4 are closely related alphaherpesviruses of horses with nucleotide sequence identity within individual homologous genes ranging from 55% to 84%, and amino acid sequence identity from 55% to 96% (Telford *et al*., ~~1992;~~ 1998). ~~The two herpesviruses~~ With the exception of EHV-1 in Iceland (Thorsteinsdóttir *et al*., 2021), the two herpesviruses are considered endemic ~~enzootic~~ in all countries in which large populations of horses are maintained as part of the cultural tradition or agricultural economy. There is no recorded evidence that the two herpesviruses of ER pose any health risks to humans working with the agents. Infection with EHV-1 is listed by WOAH and is therefore the focus of this chapter.

Viral transmission to cohort animals occurs by inhalation of aerosols of virus-laden respiratory secretions. Morbidity tends to be highest in young horses sharing the same air space. Aborted tissues and placental fluids from infected mares can contain extremely high levels of live virus and represent a major source of infection. Extensive use of vaccines has not eliminated EHV-1 infections, and the world-wide ~~annual~~ financial impact from this ~~these~~ equine pathogen~~s~~ is ~~immense~~ considerable.

In horses under 3 years of age, clinical ER usually takes the form of an acute, febrile respiratory illness that spreads rapidly through the group of animals. The virus~~es~~ infects and multiplies ~~multiply~~ in epithelial cells of the respiratory mucosa. Signs of infection become apparent 2–8 days after exposure to virus, and are characterised by fever, inappetence, depression, and nasal discharge. The severity of respiratory disease varies with the age of the horse and the level of immunity resulting from previous vaccination or natural exposure. Bi-phasic fever, viraemia and complications are more likely with EHV-1 than EHV-4. Subclinical infections with EHV-1~~/~~4 are common, even in young animals. Although mortality from uncomplicated ER is rare and complete recovery within 1–2 weeks is the normal outcome, respiratory infection is a frequent and significant cause of interrupted schedules among horses

assembled for training, racing, or other equestrian events. Fully protective immunity resulting from infection is of short duration, and convalescent animals are susceptible to reinfection by EHV-1~~/4~~ after several months. Although reinfections ~~by the two herpesviruses~~ cause less severe or clinically inapparent respiratory disease, the risks of subsequent abortion or neurological disease remain. Like other herpesviruses, EHV-1~~/4~~ causes long-lasting latent infections and latently infected horses represent a potential infection risk for other horses. Virus can be reactivated as a result of stress ~~or pregnancy~~. The greatest clinical threats to individual breeding, racing, or pleasure horse operations posed by ER are the potential abortigenic and neurological *sequelae* of EHV-1 respiratory infection. ER abortions occur annually in horse populations worldwide and may be sporadic or multiple. Foals infected *in utero* may be born alive and die within a few days of birth. EHV-1 neurological disease is less common than abortions but has been recorded all over the world with associated fatalities. Outbreaks result in movement restrictions and, sometimes, cancellation of equestrian events (Couroucé *et al*., 2023; FEI, 2021).

~~Neurological disease, also known as equine herpesvirus myeloencephalopathy, remains an infrequent but serious complication of EHV-1 infection. A single mutation in the DNA polymerase gene (ORF30) has been associated with increased risk of neurological disease, however strains without this change can also cause paralysis (Goodman *et al*., 2007; Nugent *et al*., 2006). Strain typing techniques have been employed to identify viruses carrying the neuropathic marker, and it can be helpful to be aware of an increased risk of neurological complications. However, for practical purposes strain typing is not relevant for agent identification, or international trade. Strain typing may be beneficial for implementation of biosecurity measures in the management of outbreaks of equine herpesvirus myeloencephalopathy.~~

Strain typing has been shown to be not reliable for predicting the clinical outcome of EHV-1 infection but can be useful in epidemiological investigations (Garvey *et al*., 2019; Nugent *et al*., 2006; Sutton *et al*., 2019).

EU: Strain typing has been shown to be unreliable ~~not reliable~~ for predicting the clinical outcome of EHV-1 infection but can be useful in epidemiological investigations (Garvey *et al*., 2019; Nugent *et al*., 2006; Sutton *et al*., 2019)."

B. DIAGNOSTIC TECHNIQUES

~~Both~~ EHV-1 ~~and EHV-4~~ is transmitted by the respiratory route and has ~~have~~ the potential to be highly contagious~~,~~ ~~viruses~~ particularly where large numbers of horses are housed in the same air space. EHV1 ~~and the former~~ can cause explosive outbreaks of abortion or neurological disease. Rapid diagnostic methods are therefore essential ~~useful~~ for managing the disease. Real-time polymerase chain reaction (PCR) assays are ~~widely~~ routinely used by diagnostic laboratories worldwide and are both rapid and sensitive. Real-time PCR assays ~~that allow simultaneous testing for EHV-1 and EHV-4~~ have been developed for both detection of EHV-1 and quantification of viral load ~~have been developed.~~ and have replaced virus isolation ~~has been replaced by real-time PCR~~ as the frontline diagnostic test in the majority of laboratories. ~~but~~ Virus isolation can ~~also~~ still be useful, ~~particularly for the detection of viraemia. This is also true of for~~ in cases of EHV-1-associated abortions and neonatal foal deaths, when the high level of virus in the tissues usually produces a cytopathic effect in 1–3 days. Immunohistochemical or immunofluorescent approaches are employed in some laboratories ~~can be extremely useful~~ for rapid diagnosis of EHV-induced abortion from fresh or embedded tissue ~~and are relatively straightforward~~. ~~Several other techniques based on enzyme-linked immunosorbent assay (ELISA) or nucleic acid hybridisation probes have also been described, however their use is often restricted to specialised laboratories and they are not included here.~~ Virus neutralisation (VN) and complement fixation test (CFT) are the most frequently used serological tests, and seroconversion in paired samples is considered indicative of exposure to virus by natural infection or by vaccination.

Table 1. *Test methods available for the diagnosis of ~~equine rhinopneumonitis~~ infection with EHV-1 and their purpose*

Method	Purpose					
	Population freedom from infection[a]	**Individual animal freedom from infection prior to movement[b]**	**Contribute to eradication policies[c]**	**Confirmation of clinical cases[d]**	**Prevalence of infection - surveillance[e]**	**Immune status in individual animals or populations post-vaccination[f]**
Identification of the agent[g]						
Virus isolation	–	++~~+~~	–	++	–	–
PCR	–	+++	–	+++	–	–
Direct immunofluorescence	=	=	=	++	=	=
Detection of immune response						
VN	++	++	=~~+~~	++ ~~+~~	+++	+++
ELISA	+	– ++	=~~+~~	+~~+~~	++~~+~~	+~~+~~
CFT	–	–++	–	++~~+~~	–	–+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; VN = virus neutralisation;
ELISA = enzyme-linked immunosorbent assay; CFT = complement fixation test.
[a]See Appendix 1 of this chapter for justification table for the scores giving to the tests for this purpose.
[b]See Appendix 2 of this chapter for justification table for the scores giving to the tests for this purpose.
[c]No eradication policies exist for equine rhinopneumonitis.
[d]See Appendix 4 of this chapter for justification table for the scores giving to the tests for this purpose.
[e]See Appendix 5 of this chapter for justification table for the scores giving to the tests for this purpose.
[f]See Appendix 6 of this chapter for justification table for the scores giving to the tests for this purpose.
[g]A combination of agent identification methods applied on the same clinical sample is recommended.

1. ~~Identification~~ Detection of the agent

1.1. Collection and preparation of specimens

Nasal/nasopharyngeal swabs: swab extract can be used for DNA extraction and subsequent virus detection by PCR using one of a variety of published techniques or commercially available kits (see below). Virus isolation can also be attempted from the swab extracts. To increase the chances of isolating live virus, swabs are best obtained from horses during the ~~very early, febrile stages~~ acute stage of the respiratory disease, and are collected via the nares by sampling the area with a swab of an appropriate size and length for horses. After collection, the swab should be removed and transported immediately to the virology laboratory in 3 ml of cold (not frozen) virus transport medium (e.g. phosphate buffered saline [PBS] or serum-free MEM [minimal essential medium] with antibiotics). Virus infectivity can be prolonged by the addition of bovine serum albumin, fetal calf serum or gelatine to 0.1% (w/v).

Tissue samples: total DNA can be extracted using a number of commercially available kits and used in PCR to detect viral DNA (described below in Section B.1.2.1). Virus isolation from placenta and fetal tissues from suspect cases of EHV-1 abortion is most successful when performed on aseptically collected samples of placenta, liver, lung, thymus, adrenal glands and spleen. Virus may be isolated from post-mortem cases of EHV-1 neurological disease by culture of samples of brain and spinal cord but such attempts ~~to isolate virus~~ are often unsuccessful; however, ~~they~~ these samples may be useful for PCR testing and pathological examination. Tissue samples should be transported to the laboratory and held at 4°C until

inoculated into tissue culture. Samples that cannot be processed within a few hours should be stored at –70°C.

Blood: for virus detection by PCR or isolation from blood leukocytes, collect a 10–20 ml sample of blood, using an aseptic technique in ~~citrate~~, heparin or EDTA [ethylene diamine tetra-acetic acid] anticoagulant. EDTA is the preferred anticoagulant for PCR testing in some laboratories as heparin may inhibit DNA polymerase. The samples should be transported without delay to the laboratory on ice, but not frozen.

Cerebrospinal fluid: the detection of EHV-1 DNA in cerebrospinal fluid has been reported in cases of neurological disease.

1.2. Virus detection by polymerase chain reaction

~~PCR has become the primary diagnostic method for the detection of EHV-1 and EHV-4 in clinical specimens, paraffin-embedded archival tissue, or inoculated cell cultures (Borchers & Slater, 1993; Lawrence *et al.*, 1994; O'Keefe *et al.*, 1994; Varrasso *et al.*, 2001). A variety of type-specific PCR primers have been designed to distinguish between the presence of EHV-1 and EHV-4. The correlation between PCR and virus isolation techniques for diagnosis of EHV-1 or EHV-4 is high (Varrasso *et al.*, 2001).~~ Diagnosis by PCR is rapid, sensitive, and does not depend on the presence of infectious virus in the clinical sample. ~~For diagnosis of active infection by EHV,~~ PCR methods are routinely used to detect EHV-1 DNA in nasopharyngeal swabs and tissue samples ~~most reliable with tissue samples from aborted fetuses and placental tissue or from nasopharyngeal swabs of foals and yearlings~~. They are particularly useful in explosive outbreaks of abortion, respiratory or neurological disease, in which ~~a~~ rapid identification and monitoring of the virus spread is critical for guiding management strategies, including movement restrictions. PCR examination of spinal cord and brain tissue, as well as peripheral blood mononuclear cells (PBMC), are important in seeking a diagnosis on a horse with neurological signs (Pronost *et al.*, 2012).

~~Several PCR assays have been published. A nested PCR procedure can be used to distinguish between EHV-1 and EHV-4. A sensitive protocol suitable for clinical or pathological specimens (nasal secretions, blood leukocytes, brain and spinal cord, fetal tissues, etc.) has been described by Borchers & Slater (1993). However, nested PCR methods have a high risk of laboratory cross-contamination, and sensitive rapid one-step PCR tests to detect EHV-1 and EHV-4 (e.g. Lawrence *et al.,* 1994) are preferred. The WOAH Reference Laboratories use quantitative real-time PCR assays such as those targeting heterologous sequences of major glycoprotein genes to distinguish between EHV-1 and EHV-4. A multiplex real-time PCR targeting glycoprotein B gene of EHV-1 and EHV-4 was described by Diallo *et al.* (2007). PCR protocols have been developed that can differentiate between EHV-1 strains carrying the ORF30 neuropathogenic marker, using both restriction enzyme digestion of PCR products (Fritsche & Borchers, 2011) or by quantitative real-time PCR (Allen *et al,* 2007, Smith *et al.*, 2012). Methods have also been developed to type strains for epidemiological purposes, based on the ORF68 gene (Nugent *et al.,* 2006). The WOAH Reference Laboratories employ in-house methods for strain typing, however these protocols have not yet been validated between different laboratories at an international level.~~

Real-time (or quantitative) PCR has become the method of choice for ~~many~~ the majority of diagnostic ~~tests~~ laboratories and provides rapid and sensitive detection of viral DNA. Equine post-mortem tissues from newborn and adult animals or equine fetal tissue from abortions (tissues containing lung, liver, spleen, thymus, adrenal gland and placental tissues) can be used. For respiratory samples, equine nasopharyngeal swabs or deep nasal swabs (submitted in a suitable viral transport medium), buffy coat, tracheal wash (TW) or broncho-alveolar lavage (BAL) are all suitable. DNA should be extracted using an appropriate kit or robotic system.

There is no internationally standardised real-time PCR method for EHV-1 but Table 2 summarises the primer and probe sequences for some of the most widely used assays. Type-specific PCR primers have been designed to distinguish between the presence of EHV-1 and

EHV-4. The optimised thermocycler times and temperatures are documented in the publications cited.

Table 2. *Primer and probe sequences for EHV1/4 detection by real-time PCR*

Primer	Primer sequence (5' to 3')	Target	Reference
~~Forward~~	~~CAT-GTC-AAC-GCA-CTC-CCA~~	~~EHV-1 gB~~	~~Diallo *et al.*, 2006~~
~~Reverse~~	~~GGG-TCG-GGC-GTT-TCT-GT~~		
~~Probe~~	~~FAM-CCC-TAC-GCT-GCT-CC-MGB-NFQ~~		
Forward	CAT-ACG-TCC-CTG-TCC-GAC-AGA-T	EHV-1 gB	Hussey *et al.*, 2006
Reverse	GGTACTCGGCCTTTGACGAA		
Probe	FAM-TGA-GAC-CGA-AGA-TCT-CCT-CCA-CCG-A-BHQ1		
Forward	TAT-ACT-CGC-TGA-GGA-TGG-AGA-CTT-T	EHV-1 gB	Pusterla *et al.*, 2009
Reverse	TTG-GGG-CAA-GTT-CTA-GGT-GGT-T		
Probe	6FAM-ACA-CCT-GCC-CAC-CGC-CTA-CCG		
Forward	GCG-GGC-TCT-GAC-AAC-ACA-A	EHV-1 gC	ISO 17025 accredited for the detection of EHV-1 at WOAH Reference Laboratory
Reverse	TTG-TGG-TTT-CAT-GGG-AGT-GTG-TA		
Probe	FAM-TAA-CGC-AAA-CGG-TAC-AGA-A-BHQ1		

~~*This multiplex real-time PCR test has been validated to ISO 17025 and is designed for use in a 96-well format. This can be readily combined with automatic nucleic acid extraction methods. Discrimination between EHV-1 and EHV-4 is carried out by the incorporation of type-specific dual-labelled probes based on methods published by Hussey *et al.* (2006) and Lawrence *et al.* (1994). To establish such a real-time PCR assay for diagnostic purposes, validation against blinded samples is required. Sensitivity and specificity for the assay should be determined against each target. Support for development of assays and appropriate sample panels can be obtained from the WOAH Reference Laboratories.~~ Reference material and sample panels for real-time PCR can be obtained from the WOAH Reference Laboratories.

- Point of care (POC) molecular tests

Loop-mediated isothermal amplification (LAMP) assays for the detection of EHV-1 have been described (Nemoto *et al.*, 2011). An evaluation of a hydrolysis probe-based insulated isothermal PCR (iiPCR) assay for the detection of EHV-1 showed it to have a high sensitivity and specificity compared with real-time PCR (Balasuriya *et al.*, 2017). However further validation of POC tests in the field is required.

- Molecular characterisation

Allelic discrimination real-time PCR assays identifying a single nucleotide polymorphism that was originally suggested to distinguish between neuropathogenic and non-neuro-pathogenic EHV-1 strains have been developed (Smith *et al.*, 2012). However, investigations in many countries worldwide demonstrated that the nucleotide substitution was not a reliable predictor of enhanced neuropathogenicity. Multilocus typing and whole genome sequencing are useful for molecular epidemiological studies (Garvey *et al.*, 2019; Nugent *et al.*, 2006; Sutton *et al.*, 2019).

1.3. Virus isolation

Virus isolation is no longer a routine test used for EHV-1 detection in the majority of diagnostic laboratories but is more often conducted for surveillance and research purposes. A number of cell types may be used for isolation of EHV-1 (e.g. rabbit kidney [RK-13 (AATC–CCL37)], baby hamster kidney [BHK-21], Madin–Darby bovine kidney [MDBK], pig kidney [PK-15], etc.).

RK13 cells are commonly used for this purpose. ~~For efficient primary isolation of EHV-4 from horses with respiratory disease, equine-derived cell cultures must be used. Both EHV-1 and EHV-4 may be isolated from nasopharyngeal samples using primary equine fetal kidney cells or equine fibroblasts derived from dermal (E-Derm) or lung tissue. EHV-1 can be isolated on other cell types, as will be discussed later. The nasopharyngeal swab and its accompanying transport medium are transferred into the barrel of a sterile 10 ml syringe. Using the syringe plunger, the fluid is squeezed from the swab into a sterile tube. A portion of the expressed fluid can be filtered through a sterile, 0.45 µm membrane syringe filter unit into a second sterile tube if heavy bacterial contamination is expected, but this may also lower virus titre. Recently prepared cell monolayers in tissue culture flasks are inoculated with the filtered, as well as the unfiltered, nasopharyngeal swab extract. Cell monolayers in multiwell plates incubated in a 5% CO_2 environment may also be used. Virus is allowed to attach by incubating the inoculated monolayers at 37°C. Monolayers of uninoculated control cells should be incubated in parallel.~~

~~At~~ Recently prepared cell monolayers in tissue culture flasks or plates are inoculated with nasopharyngeal swab extract or homogenised tissue: approximately 10% (w/v) pooled tissue homogenates of liver, lung, thymus, adrenal gland and spleen (from aborted fetuses/neonatal foals) or of brain and spinal cord (from cases of neurological disease). Virus is allowed to attach by incubating the ~~end of the attachment period,~~ inoculated monolayers at 37°C for 1 hour after which the inocula are removed and the monolayers are rinsed twice with PBS ~~to remove virus-neutralising antibody that may~~ or maintenance medium. Monolayers of uninoculated control cells should be ~~present in the nasopharyngeal secretions~~ incubated in parallel. After addition of supplemented maintenance medium (MEM containing 2% fetal calf serum [FCS] and twice the standard concentrations of antibiotics/antifungals [penicillin, streptomycin, gentamicin, and amphotericin B]), the flasks are incubated at 37°C in a 5% CO_2 environment.

The use of a positive control ~~virus samples~~ of relatively low titre may be used to validate the isolation procedure ~~carries the risk that this may lead~~ but should be processed separately to ~~eventual~~ avoid contamination of diagnostic specimens. ~~This risk can be minimised by using routine precautions and good laboratory technique, including the use of biosafety cabinets, inoculating positive controls after the diagnostic specimens, decontaminating the surfaces in the hood while the inoculum is adsorbing and using a positive control of relatively low titre.~~ Inoculated flasks should be inspected daily by microscopy for the appearance of characteristic herpesvirus cytopathic effect (CPE) (focal rounding, increase in refractility, and detachment of cells). Cultures exhibiting no evidence of viral CPE after 1 week of incubation should be blind-passaged into freshly prepared monolayers of cells, using small aliquots of both media and cells as the inoculum. Further blind passage is usually not productive.

~~It can be useful to inoculate samples into both non-equine and equine cells in parallel to distinguish between EHV-1 and EHV-4, since EHV-4 can cause sporadic cases of abortion. Around 10% (w/v) pooled tissue homogenates of liver, lung, thymus, and spleen (from aborted fetuses) or of central nervous system tissue (from cases of neurological disease) are used for virus isolation. These are prepared by first mincing small samples of tissue into 1 mm cubes in a sterile Petri dish with dissecting scissors, followed by macerating the tissue cubes further in serum-free culture medium with antibiotics using a homogeniser or mechanical tissue grinder. After centrifugation at 1200 ***g*** for 10 minutes, the supernatant is removed and 0.5 ml is inoculated into duplicate cell monolayers in tissue culture flasks. Following incubation of the inoculated cells at 37°C for 1.5–2 hours, the inocula are removed and the monolayers are rinsed twice with PBS or maintenance medium. After addition of 5 ml of supplemented maintenance medium, the flasks are incubated at 37°C for up to 1 week or until viral CPE is observed. Cultures exhibiting no evidence of viral CPE after 1 week of incubation should be passaged a second time into freshly prepared monolayers of cells, using small aliquots of both media and cells as the inoculum.~~

Blood samples: EHV-1 ~~and, infrequently, EHV-4~~ can be isolated from PBMC. Buffy coats may be prepared from unclotted (heparinised) blood by centrifugation at ~~600~~ 525 ***g*** for ~~15~~ 5 minutes~~, and.~~ The buffy coat is taken after the plasma has been carefully removed~~. The buffy coat is then layered onto a PBMC separating solution (Ficoll; density 1077 g/ml, commercially available) and centrifuged at 400 ***g*** for 20 minutes. The PBMC interface (without most~~

granulocytes) is and washed twice in PBS (300 ***g*** for 10 minutes) and resuspended in 1 ml three times in 3 ml MEM containing 2% FCS. As a quicker alternative method, PBMC may be collected by centrifugation directly from plasma. (525 ***g*** for 5 minutes). Following the third wash, the buffy coat is harvested and resuspended in 2.5 ml MEM containing 2% FCS. An aliquot of the rinsed cell suspension is added to each of the duplicate monolayers of equine fibroblast, equine fetal or RK-13 cell monolayers in 25 cm^2 flasks containing 8–10 ml freshly added maintenance medium. The flasks can be used for DNA extraction. For virus isolation, the resuspended cells (1 ml) are co-cultivated with freshly prepared primary equine lung or RK-13 cell suspensions (5 ml) in 25 cm^2 flasks. Confluent cell monolayers are not used. The flasks are incubated at 37°C in a 5% CO_2 environment for 3 days or until the cells have reached 90% confluence. The monolayers are then rinsed three times with 1 × PBS and supplemented with 5 ml MEM containing 2% FCS. They are incubated at 37°C for 7 days; either with or without removal of the inoculum. If PBMCs are not removed prior to incubation, CPE may be difficult to detect in the presence of the massive inoculum of leukocytes: each flask of cells is freeze–thawed after 7 for a further 4 days of incubation and the contents centrifuged at 300 ***g*** for 10 minutes. Finally, 0.5 ml of the cell-free, culture medium supernatant is transferred to freshly made cell monolayers that are just subconfluent. These are incubated and observed daily for viral CPE for at least 5–6 days. Again, samples. Samples exhibiting no evidence of viral CPE after 1 week of incubation should be passaged a second time before discarding as negative.

Virus identity may be confirmed by PCR or by immunofluorescence with specific antisera. Virus isolates from positive cultures should be submitted to a WOAH Reference Laboratory for strain characterisation and to maintain a geographically diverse archive. Further strain characterisation for surveillance purposes or detection of the neurological marker can be provided at some laboratories.

1.4. Virus detection by direct immunofluorescence

Direct immunofluorescent detection of EHV-1 antigens in samples of post-mortem tissues collected from aborted equine fetuses and the placenta provides a rapid preliminary diagnosis of herpesvirus abortion (Gunn, 1992). The diagnostic reliability of this technique approaches that of virus isolation attempts from the same tissues.

In the United States of America (USA), potent polyclonal antiserum to EHV-1, prepared in swine and conjugated with FITC, is available to veterinary diagnostic laboratories for this purpose from the National Veterinary Services Laboratories of the United States Department of Agriculture (USDA). The antiserum cross-reacts with EHV-4 and hence is not useful for serotyping, however virus typing can be conducted on any virus positive specimens by PCR.

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

1.5. Virus detection by immunoperoxidase staining

Immunohistochemical (IH) staining methods, such as immunoperoxidase, have been developed for detecting EHV-1 antigen in fixed tissues of aborted equine fetuses, placental tissues or neurologically affected horses (Schultheiss *et al.*, 1993; Whitwell *et al.*, 1992). Such techniques can be used as an alternative to immunofluorescence described above and can also be readily applied to archival frozen or fixed tissue samples. Immunohistochemical staining for EHV-1 is particularly useful for the simultaneous evaluation of morphological lesions and the identification of the virus. Immunoperoxidase staining for EHV-1/4 may also be carried out on infected cell monolayers (van Maanen *et al.*, 2000). Adequate controls must be included with each immunoperoxidase test run for evaluation of both the method specificity

and antibody specificity. ~~In one WOAH Reference Laboratory, this method is used routinely for frozen or fixed tissue, using~~ If non-specific ~~rabbit~~ polyclonal sera is used ~~raised against EHV-1. This staining method is not type-specific and therefore~~ the staining method needs to be combined with virus isolation or PCR to discriminate between EHV-1 and EHV-4~~, however it provides a useful method for rapid diagnosis of EHV-induced abortion~~.

1.6. Histopathology

Histopathological examination of sections of fixed placenta and lung, liver, spleen, adrenal gland and thymus from aborted fetuses and brain and spinal cord from neurologically affected horses should be carried out. In aborted fetuses, eosinophilic intranuclear inclusion bodies present within bronchiolar epithelium or in cells at the periphery of areas of hepatic necrosis are consistent with a diagnosis of herpesvirus infection. The characteristic microscopic lesion associated with EHV-1 neuropathy is a degenerative thrombotic vasculitis of small blood vessels in the brain or spinal cord (perivascular cuffing and infiltration by inflammatory cells, endothelial proliferation and necrosis, and thrombus formation).

2. Serological tests

EHV-1 and EHV-4 are endemic in most parts of the world and seroprevalence is high, however serological testing of paired sera can be useful for diagnosis of ER in horses. A positive diagnosis is based on the demonstration of significant increases (four-fold or greater) in antibody titres in paired sera taken during the acute and convalescent stages of the disease. The results of tests performed on sera from a single collection date are, in most cases, impossible to interpret with any degree of confidence. The initial (acute phase) serum sample should be taken as soon as possible after the onset of clinical signs, and the second (convalescent phase) serum sample should be taken 2–4 weeks later.

'Acute phase' sera from mares after abortion or from horses with EHV-1 neurological disease may already contain maximal titres of EHV-1 antibody, with no increase in titres detectable in sera collected at later dates. In such cases, serological testing of paired serum samples from clinically unaffected cohort members of the herd may prove useful for retrospective diagnosis of ER within the herd.

~~Finally, the serological detection of antibodies to EHV-1 in heart or umbilical cord blood or other fluids of equine fetuses can be of diagnostic value in cases of abortion especially when the fetus is virologically negative. The EHV-1/4 nucleic acid may be identified from these tissues by PCR.~~

Serum antibody levels to EHV-1/4 may be determined by virus neutralisation (VN) (Thomson *et al*., 1976), complement fixation tests (CFT) (Thomson *et al*., 1976) or enzyme-linked immunosorbent assay (ELISA) (Crabb & Studdert, 1995). ~~There are no internationally recognised reagents or standardised techniques for performing any of the serological tests for detection of EHV-1/4 antibody; titre determinations on the same serum may differ from one laboratory to another. Furthermore,~~ The CF and VN tests detect antibodies that are cross-reactive between EHV-1 and EHV-4. Nonetheless, the demonstration of a four-fold or greater rise in antibody titre to EHV-1 or EHV-4 during the course of a clinical illness provides serological confirmation of recent infection with one of the viruses. Commercial ELISAs that distinguish EHV-1 and EHV-4 antibodies are available but less widely used than the CF and VN tests. ~~Unlike other alphaherpesviruses, DIVA ELISAs, which have been very useful in eradication programmes for bovine rhinotracheitis and pseudorabies (Aujeszky's disease), have not been developed for EHV-1/4.~~ An ELISA using a synthetic peptide for glycoprotein E as an antigen (Andoh *et al.,* 2013) is used as DIVA[1] for horses vaccinated with a modified live EHV-1 vaccine licensed in Japan, that lacks the glycoprotein E gene.

The ~~microneutralisation test is a~~ VN and the CF tests are widely used and sensitive serological assays for detecting EHV-1/4 antibody and will thus be described here.

2.1. Virus neutralisation test

This test is most commonly performed in flat-bottom 96-well microtitre plates (tissue culture grade) using a constant dose of virus and doubling dilutions of equine test sera. At least ~~two~~

[1] DIVA: detection of infection in vaccinated animals

three replicate wells for each serum dilution are required. Heat-inactivated maintenance medium with a concentration of 2% FCS (HIMM) ~~Serum-free MEM~~ is used throughout as a diluent. Virus stocks of known titre are diluted just before use to contain 100 $TCID_{50}$ (50% tissue culture infective dose) in 25 µl. Monolayers of ~~E-Derm or~~ RK-13 cells are prepared ~~monodispersed with EDTA/trypsin and resuspended at a concentration of 5 × 10^5/ml~~. ~~Note that RK-13 cells can be used with EHV-1 but do not show CPE with EHV-4.~~ Antibody positive and negative control equine sera and controls for cell viability, virus infectivity, and test serum cytotoxicity, must be included in each assay. End-point VN titres of antibody are calculated by determining the reciprocal of the highest serum dilution that protects ≥75% ~~100%~~ of the cell monolayer from virus destruction in ~~both of~~ the replicate wells.

Serum toxicity may be encountered in samples from horses repeatedly vaccinated with a commercial vaccine prepared from EHV-1 grown up in RK-13 cells. This can give rise to difficulties in interpretation of test reactions at lower serum dilutions. The problem can be overcome using E-Derm or other non-rabbit kidney derived cell line.

2.1.1. Test procedure

A suitable test procedure is as follows:

i) Prepare semi-confluent monolayers in tissue culture microtitre plates.

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

iii) Add 40 ~~25~~ µl of HIMM ~~serum-free MEM~~ to all wells of the microtitre assay plates.

iv) For test sample titration, pipette ~~25~~ 40 µl of each test serum into ~~duplicate~~ triplicate wells of ~~both rows A and B of~~ the plate. The first two rows serve as the dilution of the test serum and the third row serves as the serum toxicity control ~~and the second row as the first dilution of the test~~. Make doubling dilutions of each serum ~~starting with row B and proceeding to the bottom of the plate~~ by sequential mixing and transfer of ~~25~~ 40 µl to each subsequent row of wells. ~~Six sera can be assayed in each plate.~~ Add 40µl of HIMM to the serum control rows.

v) Add 40 ~~25~~ µl of the appropriately diluted EHV-1 ~~or EHV-4~~ virus stock to ~~each~~ all wells (100 $TCID_{50}$/well) of the test plate except those of ~~row A, which are~~ the serum controls ~~wells~~. Note that the final serum dilutions, after addition of virus, run from a starting dilution of 1/4 ~~to 1/256~~. A separate control plate should include titration of both a negative and positive (high and low) horse ~~serum~~ sera of known titre, cell control (no virus), and a back titration of virus ~~control (no serum), and a virus titration~~ using six wells per log dilution (100 $TCID_{50}$ to 0.01 $TCID_{50}$/well) ~~calculate the actual amount of virus used in the test~~

vi) Incubate the plates for 1 hour at 37°C in 5% CO_2 atmosphere. ~~Add 50 µl of the prepared E-Derm or RK-13 cell suspension (5 × 10^5 cells/ml) in MEM/10% FCS to each well.~~

vii) Transfer 50 µl from each well of the test and control plates to the tissue culture microtitre plates.

viii) Incubate the plates for 2 ~~4~~ 5 days at 37°C in an atmosphere of 5% CO_2 in air.

ix) Examine the plates microscopically for CPE and record the results on a worksheet. Confirm the validity of the test by establishing that the working dilution of stock virus is at 100 $TCID_{50}$/well, that the (high and low) positive control sera are within one well of their pre-determined titre and that the negative control serum is negative at a 1/4 dilution. This takes approximately 72 hours. If at this stage the antigen is too weak the virus concentration may be increased by extending the incubation period up to 5 days. If the antigen is too strong the test must be repeated.

Wells are scored as positive for neutralisation of virus if ≥ 75% of the cell monolayer remains intact. The highest dilution of serum resulting in ≥ 75% neutralisation of virus (<25% CPE) in replicate wells is the end-point titre for that serum. Examine the plates microscopically for CPE and record the results on a worksheet.

x) Alternatively, the cell monolayers can be scored for CPE after fixing and staining as follows: after removal of the culture fluid, immerse the plates for 15 minutes in a solution containing 2 mg/ml crystal violet, 10% formalin, 45% methanol, and 45% water. Then, rinse the plates vigorously under a stream of running tap water. Wells containing intact cell monolayers stain blue, while monolayers destroyed by virus do not stain. ~~Verify that the cell control, positive serum control, and serum cytotoxicity control wells stain blue, that the virus control and negative serum control wells are not stained, and that the actual amount of virus added to each well is between $10^{1.5}$ and $10^{2.5}$ $TCID_{50}$. Wells are scored as positive for neutralisation of virus if 100% of the cell monolayer remains intact. The highest dilution of serum resulting in complete neutralisation of virus (no CPE) in both duplicate wells is the end-point titre for that serum.~~

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

2.2. Complement fixation test

The CFT can be used for the detection and quantification of antibodies ~~against~~ to EHV-1. The test determines whether an antigen and an antibody are capable of forming a complex. The presence of an immune complex is revealed by the detector system, which consists of guinea-pig complement and sensitised sheep red blood cells (SRBCs) coated with rabbit haemolytic serum (haemolysin). In the absence of antibodies against equine herpesvirus, no antibody/antigen complex is formed, the complement remains free in the solution and the sensitised SRBCs become lysed. In the presence of antibodies against equine herpesvirus, an antibody/antigen complex is formed, the complement becomes fixed and is therefore unable to lyse the SRBCs. They subsequently form a button at the bottom of the test well.

Guinea-pig complement, rabbit haemolytic serum, complement fixation diluent (CFD) and bovine serum albumin (BSA) can be obtained commercially. The dilution of guinea-pig complement that has activity at 3 HD (haemolytic dose) in the presence of sensitised SRBCs should be optimised. The recommended dilution of rabbit haemolytic serum (or the working dilution) is sometimes provided by the supplier. However, the optimal dilution of haemolysin should be determined with the in use reagents (complement etc.) so that the test can be performed reproducibly. The optimum concentration of antigen to be used in the test should be determined using an antigen versus antibody chequerboard technique and by testing a panel of known positive sera.

The test is performed in U bottomed microtitre plates. Paired sera should be assayed on the same plate. An antibody positive serum should be included as a control on each plate. All sera are tested on a second plate containing all components except virus to check for anti-complementary activity. A back titration of the working dilution (3 HD) of complement to 2 HD, 1 HD, 0.5 HD is set up in duplicate wells on the complement control plate (eight wells in total). An SRBC control is set up in eight wells.

2.2.3. Preparation of samples

i) Samples and controls are prepared by adding 4 volumes (600 µl) of CFD to 1 volume (150 µl) of test sera to give a 1/5 dilution.

ii) Diluted serum is inactivated for 30 minutes at 60°C to destroy the naturally occurring complement.

2.2.4. Test procedure

i) Prepare the test plate and anti-complementary plate by adding 25 µl 0.05% BSA/CFD to all wells except the first column (H).

ii) Add 50 µl of 0.05% BSA/CFD to the eight wells of the complement control (back titration).

iii) Add 75 µl of 0.05% BSA/CFD to eight wells of cell control.

iv) Add 50 µl of the diluted inactivated test serum and controls to the first well of each row on both the test and anti-complementary plates. Serial doubling dilutions are then made by transferring 25 µl across the plate and discarding the final 25 ml.

v) Place the microtitre plates on ice for addition of antigen and complement.

vi) Add 25 µl of antigen (diluted to working strength in 0.05% BSA/CFD) to the test plates.

vii) Add 25 µl of 0.05% BSA/CFD to all wells of the anti-complementary plate to compensate for lack of antigen.

viii) Add 25 µl of guinea-pig complement diluted in 0.05% BSA/CFD to 3 HD to all wells except the complement control and SRBC control.

ix) Back titrate the working dilution of 3 HD complement to 2 HD, 1 HD and 0.5 HD in 200 µl volumes. Add 25 µl of each dilution to the appropriate wells.

x) Incubate all plates at 4°C overnight.

2.2.5. Preparation and addition of sheep blood

i) SRBCs collected into Alsever's solution are washed twice in 0.05% BSA/PBS solution.

ii) Gently resuspend the SRBCs in 5–10 ml 0.05% BSA/CFD solution. Dilute to 2% SRBCS (v/v packed cells) in BSA/CFD solution.

iii) Mix the 2% SRBCs with an equal volume of BSA/CFD solution containing haemolysin at its optimal sensitising concentration to give a 1% SRBC solution. Prepare an appropriate volume of this solution by allowing 3 ml per microtitre plate.

iv) Incubate at 37°C for 10 minutes. Store the 1% sensitised SRBCs overnight at 4°C.

v) The following day, incubate the 1% sensitised SRBCs at 37°C for 30 minutes. During the final 20 minutes of this incubation, transfer the test plates from 4°C to 37°C.

vi) At the end of the 30-minute incubation, add 25 ml of 1% sensitised SRBCs to all plates. Mix on a plate shaker for 30 seconds.

vii) Incubate the plates at 37°C for 30 minutes. Shake the plates after 15 minutes and at the end of this incubation (a total of three times).

viii) Incubate the plates at 4°C for 2 hours to allow the cells to settle.

ix) Read and record the test results after 2 hours.

2.2.6. Reading results

i) Confirm the validity of the test by establishing that the working dilution of complement is at 3 HD: 100% lysis at 3 HD and 2 HD, and 50% lysis at 1 HD. Distinct buttons should be visible in the eight wells of the SRBC control.

ii) There must be 100% lysis observed at the 1/5 dilution for the negative control (<5). The antibody titre of the positive control serum must read within one well of its predetermined titre.

iii) Confirm that there are no buttons visible on the anti-complementary plates. Buttoning indicates either the presence of residual native complement in the sample or that there is a non-specific complement fixing effect occurring. Sera that show anti-complementary activity should be retested and treated as described below.

iv) In the test wells, buttoning indicates the presence of antibodies in the serum. The antibody titre is the dilution at which there is 50% buttoning and 50% lysis observed.

2.2.7. Treatment of samples showing anti-complementary activity

i) Add 50 µl of guinea-pig complement to 150 µl of the serum showing anti-complementary activity.

ii) Incubate the sample at 37°C for 30 minutes.

iii) Add 550 µl of CFD (1:5 dilution).

iv) Heat inactivate at 60°C for 30 minutes.

C. REQUIREMENTS FOR VACCINES

1. Background

Both live attenuated and inactivated vaccines are available for use in horses as licensed, commercially prepared products for use in reducing the impact of disease in horses caused by EHV-1/4 infection. The products contain different permutations of EHV-1 and EHV-4 and some also include equine influenza virus.

Clinical experience has demonstrated that vaccination can be useful for reducing clinical signs of respiratory disease and incidence of abortion, however none of the vaccines protect against neurological disease. Multiple doses repeated annually, of each of the currently marketed ER vaccines are recommended by their respective manufacturers. Vaccination schedules vary with a particular vaccine.

The indications stated on the product label for use of several available vaccines for ER are either as a preventative of herpesvirus-associated respiratory disease, or as an aid in the prevention of abortion, or both. A minority of ~~Only four~~ vaccine products have met the regulatory requirements for claiming efficacy in providing protection from herpesvirus abortion as a result of successful vaccination and challenge experiments in pregnant mares. None of the vaccine products have been demonstrated to prevent the occurrence of neurological disease sometimes associated with EHV-1 infection.

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

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

The master seed virus (MSV) for ER vaccines must be prepared from strains of EHV-1 and/or EHV-4 that have been positively and unequivocally identified ~~by both serological and genetic tests~~. Seed virus must be propagated in a cell line approved for equine vaccine production by the appropriate regulatory agency. A complete record of original source (including isolate number, location, year of isolation), passage history, medium used for propagation, etc., shall be kept for the master seed preparations of both the virus(es) and cell stock(s) intended for use in vaccine production.

2.1.1. Biological characteristics of the master seed

Permanently stored stocks of both MSV and master cell stock (MCS) used for vaccine production must be demonstrated to be pure, safe and, in the case of MSV, also immunogenic.

Generally, the fifth passage from the MSV and the twentieth passage from the MCS are the highest allowed for vaccine production. Results of all quality control tests on master seeds must be recorded and made a part of the licensee's permanent records.

2.1.2. Quality criteria

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

2.1.3. Validation as a vaccine strain

Tests for immunogenicity of the EHV-1/4 MSV stocks should be performed in horses on an experimental test vaccine prepared from the highest passage level of the MSV allowed for use in vaccine production. The test for MSV immunogenicity consists of vaccination of horses with low antibody titres (< 1:24 by VN test) to EHV-1/4, with doses of the test vaccine that will be recommended on the final product label (Goodman *et al.*, 2006; Van de Walle *et al.*, 2010). Second serum samples should be obtained and tested for significant increases in neutralising antibody titre against the virus, 21 days after the final dose.

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

2.2. Method of manufacture

2.2.1. Procedure

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

2.2.2. Requirements for ingredients

Cells, virus, culture medium, and medium supplements of animal origin that are used for the preparation of production lots of vaccine must be derived from bulk stocks that have passed the prescribed tests for bacterial, fungal, and mycoplasma sterility; nontumorgenicity; and absence of extraneous viral agents.

2.2.3. Final product batch tests

i) Sterility

Samples taken from each batch of completed vaccine are tested for bacteria, fungi, and mycoplasma contamination. Procedures to establish that the vaccine is free from extraneous viruses are also required; such tests should include inoculation of cell cultures that allow detection of the common equine viruses, as well as techniques for the detection of BVDV and PPV in ingredients of animal origin used in the production of the batch of vaccine.

ii) Identity

Identity tests shall demonstrate that no other vaccine strain is present when several strains are propagated in a laboratory used in the production of multivalent vaccines.

iii) Safety

Safety tests shall consist of detecting any abnormal local or systemic adverse reactions to the vaccine in the host species by all vaccination route(s). Tests to assure safety of each production batch of ER vaccine must demonstrate complete inactivation of virus (for inactivated vaccines) as well as a level of residual virus-killing agent that does not exceed the maximal allowable limit (e.g. 0.2% for formaldehyde).

iv) Batch potency

Batch potency is examined on the final formulated product~~. Batch control of antigenic potency for EHV-1 vaccines only may be tested by measuring the ability of dilutions of the vaccine to protect hamsters from challenge with a lethal dose of hamster-adapted EHV-1 virus.~~ Although potency testing on production batches of ER vaccine may ~~also~~ be performed by vaccination of susceptible horses followed by assay for seroconversion, ~~the recent availability of virus type-specific MAbs has permitted development of less costly and more~~ rapid *in-vitro* immunoassays exist for antigenic potency. The basis for such *in-vitro* assays for ER vaccine potency is the determination, by use of ~~the~~ specific MAb, of the presence of at least the minimal amount of viral antigen within each batch of vaccine that correlates with the required level of protection (or seroconversion rate) in a standard animal test for potency.

2.3. Requirements for authorisation/registration/licencing

2.3.1. Manufacturing process

For registration of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Sections C.2.1 and C.2.2) should be submitted to the authorities. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

2.3.2 Safety requirements

Vaccine safety should be evaluated in vaccinated animals using different assays (see Section 2.2.3.iii).

2.3.3 Efficacy requirements

Vaccine efficacy (protection) is estimated in vaccinated animals directly by evaluating their resistance to live pathogen challenge.

2.3.4 Duration of immunity

As part of the licensing or marketing authorisation procedure, the manufacturer may be required to demonstrate the duration of immunity (DOI) of a given vaccine by either challenge or alternative test at the end of the claimed period of protection.

Tests to establish the duration of immunity to EHV-1~~/4 or EHV1/4~~ achieved by immunisation with each batch of vaccine are not required. The results of many reported observations indicate that immunity induced by vaccination-against EHV-1~~ or EHV induced immunity to EHV-1/4~~ is not more than a few months in duration; these observations are reflected in the frequency of revaccination recommended on ER vaccine product labels.

2.3.5 Stability

As part of the licensing or marketing authorisation procedure, the manufacturer will be required to demonstrate the stability of all the vaccine's properties at the end of the claimed shelf-life period. Storage temperature shall be indicated, and warnings should be given if product is damaged by freezing or ambient temperature.

At least three production batches of vaccine should be tested for shelf life before reaching a conclusion on the vaccine's stability. When stored at 4°C, inactivated vaccine products generally maintain their original antigenic potency for at least 1 year. Lyophilised preparations of the live virus vaccine are also stable during storage for 1 year at 4°C. Following reconstitution, live virus vaccine is unstable and cannot be stored without loss of potency.

Note: current vaccines are authorised for prevention of respiratory disease or as an aid in the prevention of abortion. Unless the vaccine's ability to prevent neurological disease is under investigation, the virus used in the challenge experiments should not be a strain with a history of inducing neurological disease.

REFERENCES

ANDOH K., TAKASUGI M., MAHMOUD H.Y., HATTORI S., TERADA Y., NOGUCHI K., SHIMODA H., BANNAI H., TSUJIMURA K., MATSUMURA T., KONDO T. & MAEDA K. (2013). Identification of a major immunogenic region of equine herpesvirus-1 glycoprotein E and its application to enzyme-linked immunosorbent assay. *Vet. Microbiol.*, **164**, 18–26.

~~ALLEN G.P. (2007). Development of a real-time polymerase chain reaction assay for rapid diagnosis of neuropathogenic strains of equine herpesvirus-1. *J Vet. Diagn. Invest.*, **19**, 69–72.~~

~~ALLEN G.P. & BRYANS J.T. (1986). Molecular epidemiology, pathogenesis and prophylaxis of equine herpesvirus-1 infections. *In:* Progress in Veterinary Microbiology and Immunology, Vol. 2, Pandey R., ed. Karger, Basel, Switzerland & New York, USA, 78–144.~~

ALLEN G.P., KYDD J.H., SLATER J.D. & SMITH K.C. (1999). Recent advances in understanding the pathogenesis, epidemiology, and immunological control of equid herpesvirus-1 (EHV-1) abortion. *Equine Infect. Dis.*, **8,** 129–146.

ALLKOFER A., GARVEY M., RYAN E., LYONS R., RYAN M., LUKASEVICIUTE G., WALSH C., VENNER M. & CULLINANE A. (2021). Primary vaccination in foals: a comparison of the serological response to equine influenza and equine herpesvirus vaccines administered concurrently or 2 weeks apart. *Arch. Virol.*, **166**, 571–579.

ATASEVEN V.S., DAĞALP S.B., GÜZEL M., BAŞARAN Z., TAN M.T. & GERAGHTY B. (2009). Prevalence of equine herpesvirus-1 and equine herpesvirus-4 infections in equidae species in Turkey as determined by ELISA and multiplex nested PCR. *Res. Vet. Sci.*, **86**, 339–344.

BALASURIYA U.B.R., LEE P.-Y.A., TSAI Y.-L., TSAI C.-F., SHEN Y.-H., CHANG H.-F. G., SKILLMAN A., WANG H.-T.T., PRONOST S. & ZHANG Y. (2017). Translation of a laboratory-validated equine herpesvirus-1 specific real-time PCR assay into an insulated isothermal polymerase chain reaction (iiPCR) assay for point-of-need diagnosis using POCKIT™ nucleic acid analyzer. *J. Virol. Methods*, **241**, 58–63.

BANNAI H., MAE N., ODE H., NEMOTO M., TSUJIMURA K., YAMANAKA T., KONDO T. & MATSUMURA T. (2014). Successful control of winter pyrexias caused by equine herpesvirus type 1 in Japanese training centers by achieving high vaccination coverage. *Clin. Vaccine Immunol.*, **21**, 1070–1076.

BANNAI H., TSUJIMURA K., NEMOTO M., OHTA M., YAMANAKA T., KOKADO H. & MATSUMURA T. (2019). Epizootiological investigation of equine herpesvirus type 1 infection among Japanese racehorses before and after the replacement of an inactivated vaccine with a modified live vaccine. *BMC Vet. Res.*, **15**, 280.

BARBIC L., LOJKIC I., STEVANOVIC V., BEDEKOVIC T., STARESINA V., LEMO N., LOJKIC M. & MADIC J. (2012). Two outbreaks of neuropathogenic equine herpesvirus type 1 with breed-dependent clinical signs. *Vet. Rec.*, **170**, 227.

BRESGEN C., LÄMMER M., WAGNER B., OSTERRIEDER N. & DAMIANI A.M. (2012). Serological responses and clinical outcome after vaccination of mares and foals with equine herpesvirus type 1 and 4 (EHV-1 and EHV-4) vaccines. *Vet. Microbiol.*, **160**, 9–16.

COUROUCÉ A., NORMAND C., TESSIER C., POMARES R., THÉVENOT J., MARCILLAUD-PITEL C., LEGRAND L., PITEL P.H., PRONOST S. & LUPO C. (2023). Equine Herpesvirus-1 Outbreak During a Show-Jumping Competition: A Clinical and Epidemiological Study. *J. Equine Vet. Sci.*, **128**, 104869.

~~BORCHERS K. & SLATER J. (1993). A nested PCR for the detection and differentiation of EHV-1 and EHV-4. *J. Virol. Methods*, **45**, 331–336.~~

~~BRYANS J.T. & ALLEN G.P. (1988). Herpesviral diseases of the horse. *In:* Herpesvirus Diseases of Animals, Wittman G., ed. Kluwer, Boston, USA, 176–229.~~

~~CRABB B.S., MACPHERSON C.M., REUBEL G.H., BROWNING G.F., STUDDERT M.J. & DRUMMER H.E. (1995). A type-specific serological test to distinguish antibodies to equine herpesviruses 4 and 1. *Arch. Virol.*, **140**, 245–258.~~

CRABB B.S. & STUDDERT M.J. (1995). Equine herpesviruses 4 (equine rhinopneumonitis virus) and 1 (equine abortion virus). *Adv. Virus Res.*, **45**, 153–190.

~~DIALLO I.S., HEWITSON G., WRIGHT L., RODWELL B.J. & CORNEY B.G. (2006). Detection of equine herpesvirus type 1 using a real-time polymerase chain reaction. *J. Virol. Methods,* **131**, 92–98.~~

~~DIALLO I.S., HEWITSON G., WRIGHT L.L., KELLY M.A., RODWELL B.J. & CORNEY B.G. (2007). Multiplex real-time PCR for detection and differentiation of equid herpesvirus 1 (EHV-1) and equid herpesvirus 4 (EHV-4). *Vet. Microbiol.*, **123**, 93–103.~~

EL BRINI Z., FASSI FIHRI O. & PAILLOT R. (2021). Seroprevalence of Equine Herpesvirus 1 (EHV-1) and Equine Herpesvirus 4 (EHV-4) in the Northern Moroccan Horse Populations. *Animals* (*Basel*), **11**, 2851.

FÉDÉRATION EQUESTRE INTERNATIONALE (FEI) (2021), Management of an EHV-1 outbreak at FEI events and its international impact. *Vet. Rec.,* **189**, e905. https://doi.org/10.1002/vetr.905

FOOTE C.E., GILKERSON J.R., WHALLEY J.M. & LOVE D.N. (2003). Seroprevalence of equine herpesvirus 1 in mares and foals on a large Hunter Valley stud farm in years pre- and postvaccination. *Aust. Vet. J.*, **81**, 283–288.

GARVEY M., LYONS R. & HECTOR R.D. (2019). Molecular Characterisation of Equine Herpesvirus 1 Isolates from Cases of Abortion, Respiratory and Neurological Disease in Ireland between 1990 and 2017. *Pathogens*, **8**, 7.

GILDEA S., SANCHEZ HIGGINS M.J., JOHNSON G., WALSH C. & CULLINANE A. (2016). Concurrent vaccination against equine influenza and equine herpesvirus – a practical approach. *Influenza Other Respir. Viruses*, **10**, 433–437.

GILKERSON J. R., WHALLEY J. M., DRUMMER H. E., STUDDERT M.J. & LOVE D.N. (1999). Epidemiology of EHV-1 and EHV-4 in the mare and foal populations on a Hunter Valley stud farm: are mares the source of EHV-1 for unweaned foals. *Vet. Microbiol.,* **68**, 27–34.

GOODMAN L.B., WAGNER B., FLAMINIO M.J., SUSSMAN K.H., METZGER S.M., HOLLAND R. & OSTERRIEDER N. (2006). Comparison of the efficacy of inactivated combination and modified-live virus vaccines against challenge infection with neuropathogenic equine herpesvirus type 1 (EHV-1). *Vaccine*, **24**, 3636–3645.

GRYSPEERDT A., VANDERKERCKHOVE A., VAN DOORSSELAERE J., VAN DE WALLE G., & NAUWYNCK H. (2011). Description of an unusually large outbreak of nervous system disorders caused by equine herpesvirus 1 (EHV1) in 2009 in Belgium. *Vlaams Diergeneeskundig Tijdschrif*, **80**, 147–153.

~~FRITSCHE A.K. & BORCHERS K. (2011). Detection of neuropathogenic strains of equid herpesvirus 1 (EHV-1) associated with abortions in Germany. *Vet. Microbiol.,* **147**, 176-180.~~

GUNN H.M. (1992). A direct fluorescent antibody technique to diagnose abortion caused by equine herpesvirus. *Irish Vet. J.*, **44**, 37–40.

HELDENS J.G., HANNANT D., CULLINANE A.A., PRENDERGAST M.J., MUMFORD J.A., NELLY M., KYDD J.H., WESTSTRATE M.W. & VAN DEN HOVEN R.(2001). Clinical and virological evaluation of the efficacy of an inactivated EHV1 and EHV4 whole virus vaccine (Duvaxyn EHV1,4). Vaccination/challenge experiments in foals and pregnant mares. *Vaccine*, **19**, 4307–4317.

HENNINGER R.W., REED S.M., SAVILLE W.J., ALLEN G.P., HASS G.F., KOHN C.W. & SOFALY C. (2007). Outbreak of neurologic disease caused by equine herpesvirus-1 at a university equestrian center. *J. Vet. Intern. Med.,* **21**, 157–165.

~~GOODMAN L.B., LOREGIAN A., PERKINS G.A., NUGENT J., BUCKLES E.L., MERCORELLI B., KYDD J.H., PALÙ G., SMITH K.C., OSTERRIEDER N. & DAVIS-POYNTER N. (2007). A point mutation in a herpesvirus polymerase determines neuropathogenicity. *PLoS Pathog*., **3** (11), e160.~~

~~HARTLEY C.A., WILKS C.R., STUDDERT M.J. & GILKERSON J.R. (2005). Comparison of antibody detection assays for the diagnosis of equine herpesvirus 1 and 4 infections in horses. *Am. J. Vet. Res*., **66**, 921–928.~~

HUSSEY S.B., CLARK R., LUNN K.F., BREATHNACH C., SOBOLL G., WHALLEY J.M. & LUNN D.P. (2006). Detection and quantification of equine herpesvirus-1 viremia and nasal shedding by real-time polymerase chain reaction. *J. Vet. Diagn. Invest*., **18**, 335–342.

KYDD J. H., WATTRANG E. & HANNANT D. (2003). Pre-infection frequencies of equine herpesvirus-1 specific, cytotoxic T lymphocytes correlate with protection against abortion following experimental infection of pregnant mares. *Vet. Immunol. Immunopathol*., **96**, 207–217.

LAWRENCE G.L., GILKERSON J., LOVE D.N., SABINE M. & WHALLEY J.M. (1994). Rapid, single-step differentiation of equid herpesvirus 1 and 4 from clinical material using the polymerase chain reaction and virus-specific primers. *J. Virol. Methods*, **47**, 59–72.

MCCARTAN C.G., RUSSELL M.M., WOOD J.L. & MUMFORD J.A. (1995). Clinical, serological and virological characteristics of an outbreak of paresis and neonatal foal disease due to equine herpesvirus-1 on a stud farm. *Vet. Rec.*, **136**, 7–12.

MOORE, S.E., Strang, C.L., Marr, C.M., Newton, R. and Cameron, I.J. (2019), Management of an outbreak of multiple equine herpesvirus type 1 abortions among vaccinated mares on a large UK Thoroughbred stud farm. Vet Rec Case Rep, 7: e000799.

NEMOTO M., OHTA M., TSUJIMURA K., BANNAI H., YAMANAKA T., KONDO T. & MATSUMURA T. (2011). Direct detection of equine herpesvirus type 1 DNA in nasal swabs by loop-mediated isothermal amplification (LAMP). *J. Vet. Med. Sci.,* **73**, 1225–1227.

NUGENT J., BIRCH-MACHIN I., SMITH K.C., MUMFORD J.A., SWANN Z., NEWTON J.R., BOWDEN R.J., ALLEN G.P. & DAVIS-POYNTER N. (2006). Analysis of equid herpesvirus 1 strain variation reveals a point mutation of the DNA polymerase strongly associated with neuropathogenic versus nonneuropathogenic disease outbreaks. *J. Virol.,* **80**, 4047–4060.

PRONOST S., LEGRAND L., PITEL P.H., WEGGE B., LISSENS J., FREYMUTH F., RICHARD E. & FORTIER G. (2012). Outbreak of equine herpesvirus myeloencephalopathy in France: a clinical and molecular investigation. *Transbound. Emerg. Dis*., **59**, 256–263.

PUSTERLA N., KALSCHEUR M., PETERS D., BIDWELL L., HOLTZ S., BARNUM S. & LAWTON K. (2023). Investigation of the Frequency of Detection of Common Respiratory Pathogens in Nasal Secretions and Environment of Healthy Sport Horses Attending a Multi-Week Show Event during the Summer Months. *Viruses*, **15**, 1225.

PUSTERLA N., SANDLER-BURTNESS E., BARNUM S., HILL L.A., MENDONSA E., KHAN R., PORTENER D., RIDLAND H. & SCHUMACHER S. (2022). Frequency of Detection of Respiratory Pathogens in Nasal Secretions From Healthy Sport Horses Attending a Spring Show in California. *J. Equine Vet. Sci,* **117**, 104089.

PUSTERLA N., WILSON W.D., MAPES S., FINNO C., ISBELL D., ARTHUR R.M. & FERRARO G.L. (2009). Characterization of viral loads, strain and state of equine herpesvirus-1 using real-time PCR in horses following natural exposure at a racetrack in California. *Vet. J.,* **179**, 230–239.

O'KEEFE J.S., JULIAN A., MORIARTY K., MURRAY A. & WILKS C.R. (1994). A comparison of the polymerase chain reaction with standard laboratory methods for the detection of EHV-1 and EHV-4 in archival tissue samples. *N.Z. Vet. J.*, **42**, 93–96.

SCHULTHEISS P.C., COLLINS J.K. & CARMAN J. (1993). Use of an immunoperoxidase technique to detect equine herpesvirus-1 antigen in formalin-fixed paraffin-embedded equine fetal tissues. *J. Vet. Diagn. Invest*., **5**, 12–15.

Singh B.K., Ahuja S. & Gulati B.R. (2004). Development of a neutralizing monoclonal antibody-based blocking ELISA for detection of equine herpesvirus 1 antibodies. *Vet. Res .Commun*., **28**, 437–446.

SMITH F.L., WATSON J.L., SPIER S.J., KILCOYNE I., MAPES S., SONDER C. & PUSTERLA N. (2018). Frequency of shedding of respiratory pathogens in horses recently imported to the United States. *J. Vet. Intern. Med*., **32**, 1436–1441.

SMITH K.L., LI Y., BREHENY P., COOK R.F., HENNEY P.J., SELLS S., PRONOST S., LU Z., CROSSLEY B.M., TIMONEY P.J. & BALASURIYA U.B. (2012). Development and validation of a new and improved allelic discrimination real-time PCR assay for the detection of equine herpesvirus-1 (EHV-1) and differentiation of A2254 from G2254 strains in clinical specimens. *J. Clin. Microbiol.*, **50**, 1981–1988.

STRANG C. & NEWTON R. (2017). Control and disease clearance after neurological EHV-1 in the UK. *Vet. Rec*., **181**, 678–679.

SUTTON G., GARVEY M., CULLINANE A., JOURDAN M., FORTIER C., MOREAU P., FOURSIN M., GRYSPEERDT A., MAISONNIER V., MARCILLAUD-PITEL C., LEGRAND L., PAILLOT R. & PRONOST S. (2019). Molecular Surveillance of EHV-1 Strains Circulating in France during and after the Major 2009 Outbreak in Normandy Involving Respiratory Infection, Neurological Disorder, and Abortion. *Viruses*, **11**, 916.

TELFORD E.A.R., WATSON M.S., MCBRIDE K. & DAVISON A.J. (1992). The DNA sequence of equine herpesvirus-1. *Virology*, **189**, 304–316.

TELFORD E.A.R., WATSON M.S., PERRY J., CULLINANE A.A. & DAVISON A.J. (1998). The DNA sequence of equine herpesvirus 4. *J. Gen. Virol.,* **79***,* 1197–1203.

THOMSON G.R., MUMFORD J.A., CAMPBELL J., GRIFFITHS L. & CLAPHAM P. (1976). Serological detection of equid herpesvirus 1 infections of the respiratory tract. *Equine Vet. J*., **8***,* 58–65.

THORSTEINSDÓTTIR L., GUÐMUNDSSON G., JENSSON H., TORSTEINSDÓTTIR S. & SVANSSON V. (2021). Isolation of equid alphaherpesvirus 3 from a horse in Iceland with equine coital exanthema. *Acta Vet. Scand.*, **63**, 6.

VAN MAANEN C., VREESWIJK J., MOONEN P., BRINKHOF J. DE BOER-LUIJTZE E. & TERPSTRA C. (2000). Differentiation and genomic and antigenic variation among fetal, respiratory, and neurological isolates from EHV1 and EHV4 infections in The Netherlands. *Vet. Q.*, **22**, 88–93.

VAN MAANEN C., WILLINK D.L., SMEENK L.A., BRINKHOF J. & TERPSTRA C. (2000). An equine herpesvirus 1 (EHV1) abortion storm at a riding school. *Vet. Q.*, **22**, 83–87.

VAN DE WALLE G.R., MAY M.A., PETERS S.T., METZGER S.M., ROSAS C.T. & OSTERRIEDER N. (2010). A vectored equine herpesvirus type 1 (EHV-1) vaccine elicits protective immune responses against EHV-1 and H3N8 equine influenza virus. *Vaccine*, **28**, 1048–1055. doi: 10.1016/j.vaccine.2009.10.123. Epub 2009 Nov 6.

~~VARRASSO A., DYNON K., FICORILLI N., HARTLEY C.A., STUDDERT M.J. & DRUMMER H.E. (2001). Identification of equine herpesviruses 1 and 4 by polymerase chain reaction. *Aust. Vet. J.*, **79**, 563–569.~~

WHITWELL K.E., GOWER S.M. & SMITH K.C. (1992). An immunoperosidase method applied to the diagnosis of equine herpesvirus abortion, using conventional and rapid microwave techniques. *Equine Vet. J.*, **24**, 10–12.

YASUNAGA S., MAEDA K., MATSUMURA T., KONDO T. & KAI K. (2000). Application of a type-specific enzyme-linked immunosorbent assay for equine herpesvirus types 1 and 4 (EHV-1 and -4) to horse populations inoculated with inactivated EHV-1 vaccine. *J. Vet. Med. Sci.*, **62**, 687–691.

*

* *

NB: There are WOAH Reference Laboratories for equine rhinopneumonitis (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 equine rhinopneumonitis and to submit strains for further characterisation.

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2017.

Annex 14. Chapter 3.8.1. 'Border disease'

SECTION 3.8.

~~OVIDAE AND~~ CAPRINAE

CHAPTER 3.8.1.

BORDER DISEASE

SUMMARY

Border disease (BD) is a viral disease of sheep and goats first reported in sheep in 1959 from the border region of England and Wales, and since recorded world-wide. Prevalence rates in sheep vary from 5% to 50% between countries and from region to region within countries. Clinical signs include barren ewes, abortions, stillbirths and the birth of small weak lambs. Affected lambs can show ~~and~~ a fine tremor, abnormal body conformation and hairy fleeces (so-called 'hairy-shaker' or 'fuzzy' lambs). Consequently, the disease has sometimes been referred to as 'hairy shaker disease'. Vertical transmission plays an important role in the epidemiology of the disease. Infection of fetuses can result in the birth of persistently infected (PI) lambs. These PI lambs are viraemic, antibody negative and constantly excrete virus. The virus spreads from sheep to sheep, with PI animals being the most potent source of infection. Infection in goats is less common with abortion being the main presenting sign.

BD is caused by the Pestivirus *border disease virus (BDV), but in some parts of the world, especially where there is close contact between sheep or goats and cattle, the same clinical signs may be caused by infection with bovine viral diarrhoea virus (BVDV). Therefore the genetic and antigenic differences between BDV and BVDV need to be taken into consideration when investigating disease outbreaks or certifying animals or germplasm for international movement. It is important to identify the viraemic PI animals so that they will not be used for breeding or trading purposes. Serological testing is insufficient. However, it is generally considered that serologically positive, nonviraemic sheep ~~are 'safe',~~ do not present a risk as latent infections are not known to occur in recovered animals. Pregnant seropositive, nonviraemic animals may, however, present a risk by carrying a PI fetus that cannot be detected until after parturition.*

Identification of the agent: *BDV is a species of* Pestivirus (Pestivirus ovis) *in the family* Flaviviridae *and is closely related to classical swine fever virus* (Pestivirus suis) *and BVDV viruses, which are classified in the distinct species:* Pestivirus bovis (*commonly known as BVDV type 1*), Pestivirus tauri (*formerly BVDV type 2*) *and* Pestivirus brazilense (*BVDV type 3 or Hobi-like pestivirus*). *Nearly all isolates of BDV are noncytopathogenic in cell culture. There are no defined serotypes but virus isolates exhibit considerable antigenic diversity. A number of separate genotypes have been identified.*

Apparently healthy PI sheep resulting from congenital infection can be identified by direct detection of virus or nucleic acid in blood or tissues or by virus isolation in cell culture followed by immunostaining to detect the noncytopathogenic virus.

Diagnostic methods: *The demonstration of virus by culture and antigen detection may be less reliable in lambs younger than 2 months that have received colostral antibody. Acute infection is usually subclinical and viraemia is transient and difficult to detect. The isolation of virus from*

tissues of aborted or stillborn lambs is often difficult but virus can be detected by sensitive reverse transcriptase polymerase chain reaction methods that are able to detect residual nucleic acid. However, tissues and blood from PI sheep more than a few months old contain high levels of virus, which can be easily identified by isolation and direct methods to detect antigens or nucleic acids. As sheep may be infected with BVDV, it is preferable to use diagnostic assays that are 'pan-pestivirus' reactive and will readily detect all strains of BDV and BVDV.

***Serological tests:** Acute infection with BDV is best confirmed by demonstrating seroconversion using paired or sequential samples from several animals in the group. The enzyme-linked immunosorbent assay and virus neutralisation test (VNT) are the most commonly used antibody detection methods. Due to the antigenic differences between BDV and BVDV, assays for the detection of antibodies to BDV, especially by VNT, should preferably be based on a strain of BDV.*

***Requirements for vaccines:** There is no standard vaccine for BDV, but a commercial killed whole-virus vaccine has been produced. Ideally, such a vaccine should be suitable for administration to females before breeding for prevention of transplacental infection. The use of BVDV vaccines has been advocated, but the antigenic diversity of BD viruses must be considered. In many instances, the antigenic diversity of BDV strains is sufficiently different to BVDV that a BVDV vaccine is unlikely to provide protection.*

BD viruses have contaminated several modified live veterinary vaccines produced in sheep cells or containing sheep serum. This potential hazard should be recognised by manufacturers of biological products.

A. INTRODUCTION

Border disease virus (BDV) is a *Pestivirus* of the family *Flaviviridae* and is closely related to classical swine fever virus (CSFV) and bovine viral diarrhoea virus (BVDV). There are ~~four~~ a number of officially recognised species, namely – BDV (*Pestivirus ovis*) CSFV (*Pestivirus suis*), BVDV types 1 and 2 (taxonomically known as *Pestivirus bovis* and *Pestivirus tauri*, respectively) and ~~BDV (ICTV, 2016)~~ BVDV 3 or Hobi-like pestivirus (*Pestivirus brazilense*) (Postler *et al.*, 2023), but a number of other pestiviruses that are considered to be distinct species have been reported. While CSF viruses are predominantly restricted to pigs, ~~examples of~~ there are situations where the other ~~three~~ species have ~~all~~ been recovered from sheep. While the majority of isolates have been identified as BD viruses in areas where sheep or goats are raised in isolation from other species (Vilcek *et al.*, 1997), in regions where there is close contact between small ruminants and cattle, BVDV may be frequently identified (Carlsson, 1991). Nearly all virus isolates of BDV are noncytopathogenic, although occasional cytopathic viruses have been isolated (Vantsis *et al.*, 1976). BDV spreads naturally among sheep by the oro-nasal route and by vertical transmission. It is principally a cause of congenital disease in sheep and goats, but can also cause acute and persistent infections. Infection is less common in goats, in which persistent infection is rare as abortion is the main presenting sign. Pigs may also be infected by pestiviruses other than CSFV and antibodies to BDV in pigs may interfere with tests for the diagnosis of CSF (Oguzoglu *et al.*, 2001). Several genotypes of BD viruses from sheep, goats and Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*) have been described. Phylogenetic analysis using computer-assisted nucleotide sequence analysis suggests that genetic variability among BD viruses is greater than within each of the other *Pestivirus* species. ~~Four distinguishable genogroups of BDV have been described as well as putative novel *Pestivirus* genotypes from Tunisian sheep and a goat~~ At least eight BDV genotypes have been described (BDV type 1 to BDV type 8). Other ovine pestiviruses have been identified that are responsible for BD-like syndromes such as Tunisian and Tunisian-like, Aydin-like (Pestivirus I, Turkey) Pestivirus genotypes from Tunisian sheep and a goat and a new emerging ovine pestivirus (OVPV) that were found to be genetically and antigenically closely related to CSFV (Becher *et al.*, 2003; Righi *et al.*, 2021; Vilcek & Nettleton, 2006). The chamois BDV is similar to isolates from sheep in the Iberian Peninsula (Valdazo-Gonzalez *et al.*, 2007). This chapter describes BDV infection in sheep. Chapter 3.4.7 *Bovine viral diarrhoea* should also be consulted for related diagnostic methods.

1. Acute infections

Healthy newborn and adult sheep exposed to BDV usually experience only mild or inapparent disease. Slight fever and a mild leukopenia are associated with a short-lived viraemia detectable between days 4 and 11 post-infection, after which virus neutralising antibody appears in the serum (Thabti *et al.*, 2002).

Acute infections are best diagnosed serologically using paired sera from a representative number of sheep. Occasional BDV isolates have been shown to produce high fever, profound and prolonged leukopenia, anorexia, conjunctivitis, nasal discharge, dyspnoea and diarrhoea, and 50% mortality in young lambs. One such isolate was recovered from a severe epidemic of BD among dairy sheep in 1984 (Chappuis *et al*., 1986). A second such isolate was a BDV contaminant of a live CSFV vaccine (Wensvoort & Terpstra, 1988).

2. Fetal infection

The main clinical signs of BD are seen following the infection of pregnant ewes. While the initial maternal infection is subclinical or mild, the consequences for the fetus are serious. Fetal death may occur at any stage of pregnancy, but is more common in fetuses infected early in gestation. Small dead fetuses may be resorbed or their abortion may pass unnoticed as the ewes continue to feed well and show no sign of discomfort. As lambing time approaches, the abortion of larger fetuses, stillbirths and the premature births of small, weak lambs will be seen. Confirmation that an abortion or stillbirth is due to BDV is often difficult to establish, but virus may be isolated from fetal tissues in some cases. The use of an appropriate real-time reverse-transcription polymerase chain reaction (RT-PCR) assay may give a higher level of success because of the advantages of high sensitivity and the ability to detect genome from non-infectious virus. In aborted fetuses, it is also possible to detect virus by immunohistochemistry of brain, thyroid and other tissues (Thur *et al*., 1997). Samples of fetal fluids or serum should be tested for BDV antibody.

During lambing, an excessive number of barren ewes will become apparent, but it is the diseased live lambs that present the main clinical features characteristic of BD. The clinical signs exhibited by BD lambs are very variable and depend on the breed of sheep, the virulence of the virus and the time at which infection was introduced into the flock. Affected lambs are usually small and weak, many being unable to stand. Nervous signs and fleece changes are often apparent. The nervous signs of BD are its most characteristic feature. The tremor can vary from violent rhythmic contractions of the muscles of the hindlegs and back, to barely detectable fine trembling of the head, ears, and tail. Fleece abnormalities are most obvious in smooth-coated breeds, which develop hairy fleeces, especially on the neck and back. Abnormal brown or black pigmentation of the fleece may also be seen in BD-affected lambs. Blood samples to be tested for the presence of BDV or antibody should be collected into anticoagulant from suspect lambs before they have received colostrum. Once lambs have ingested colostrum, it is difficult to isolate virus until they are 2 months old and maternal antibody levels have waned. However, during this period, it may be possible to detect viral antigen in skin biopsies, by immunohistochemistry, in washed leukocytes by enzyme-linked immunosorbent assay (ELISA) or by real-time RT-PCR. ELISAs directed at detection of the Erns antigen appear to be less prone to interference by maternal antibodies and can often be used to detect antigen in serum.

With careful nursing, a proportion of BD lambs can be reared, although deaths may occur at any age. The nervous signs gradually decline and can disappear by 3–6 months of age. Weakness, and swaying of the hind-quarters, together with fine trembling of the head, may reappear at times of stress. Affected lambs often grow slowly and under normal field conditions many will die before or around weaning time. In cases where losses at lambing time have been low and no lambs with obvious signs of BD have been born, this can be the first presenting sign of disease.

Some fetal infections occurring around mid-gestation can result in lambs with severe nervous signs, locomotor disturbances and abnormal skeletons. Such lambs have lesions of cerebellar hypoplasia and dysplasia, hydranencephaly and porencephaly resulting from necrotising inflammation. The severe destructive lesions appear to be immune mediated, and lambs with such lesions frequently have high titres of serum antibody to BDV. Most lambs infected in late gestation are normal and healthy and are born free from virus but with BDV antibody. Some such lambs can be weak and may die in early life (Barlow & Patterson, 1982).

3. Persistent viraemia

When fetuses survive an infection that occurs before the onset of immune competence, they are born with a persistent viraemia. The ovine fetus can first respond to an antigenic stimulus between approximately 60 and 85 days of its 150-day gestation period. In fetuses infected before the onset of immune competence, viral replication is uncontrolled and 50% fetal death is common. In lambs surviving infection in early gestation, virus is widespread in all organs. Such lambs appear to be tolerant of the virus and have a persistent infection, usually for life. A precolostral blood sample will be virus positive and antibody negative. Typically, there is no inflammatory reaction and the most characteristic pathological changes are in the central nervous system

(CNS) and skin. Throughout the CNS, there is a deficiency of myelin, and this causes the nervous signs. In the skin, primary wool follicles increase in size and the number of secondary wool follicles decreases, causing the hairy or coarse fleece.

Persistently viraemic sheep can be identified by the detection of viral antigens, nucleic acids or infectious virus in a blood sample. Viraemia is readily detectable by testing of serum at any time except within the first 2 months of life, when virus may be masked by colostral antibody and, possibly, in animals older than 4 years, some of which develop low levels of anti-BDV antibody (Nettleton *et al*., 1992). Methods other than virus isolation may be preferred to avoid interference from antibodies. When the presence of colostral antibodies is suspected, the virus may be detected in washed leukocytes and in skin by using sensitive ELISAs. Although virus detection in blood during an acute infection is difficult, persistent viraemia should be confirmed by retesting animals after an interval of at least 3 weeks. The use of real-time RT-PCR should be considered at all times and for any sample type due to its high analytical sensitivity and the lack of interference from antibodies in a sample.

Some viraemic sheep survive to sexual maturity and are used for breeding. Lambs born to these infected dams are always persistently viraemic. Persistently viraemic sheep are a continual source of infectious virus to other animals and their identification is a major factor in any control programme. Sheep being traded should be screened for the absence of BDV viraemia.

Usually persistently infected (PI) rams have poor quality, highly infective semen and reduced fertility. All rams used for breeding should be screened for persistent BDV infection on a blood sample. Semen samples can also be screened for virus, but virus isolation is much less satisfactory than from blood because of the toxicity of semen for cell cultures. Real-time RT-PCR for detection of pestivirus nucleic acid would usually overcome toxicity problems, and thus this assay should be useful for testing semen from rams.

4. Late-onset disease in persistently viraemic sheep

Some PI sheep housed apart from other animals spontaneously develop intractable diarrhoea, wasting, excessive ocular and nasal discharges, sometimes with respiratory distress. At necropsy such sheep have gross thickening of the distal ileum, caecum and colon resulting from focal hyperplastic enteropathy. Cytopathic BDV can be recovered from the gut of these lambs. With no obvious outside source of cytopathic virus, it is most likely that such virus originates from the lamb's own virus pool, similar to what occurs with BVDV. Other PI sheep in the group ~~do~~ may not develop the disease. This syndrome, which has been produced experimentally and recognised in occasional field outbreaks of BD, has several similarities with bovine mucosal disease (Nettleton *et al*., 1992).

B. DIAGNOSTIC TECHNIQUES

Table 1. *Test methods available for diagnosis of border disease and their purpose*

Method	Purpose					
	Population freedom from infection	**Individual animal freedom from infection prior to movement**	**Contribute to eradication policies**	**Confirmation of clinical cases**	**Prevalence of infection – surveillance**	**Immune status in individual animals or populations post-vaccination**
Identification of the agent[(a)]						
Virus isolation	+	++	++	+++	–	–
Antigen detection by ELISA	+	++	+++	+++	–	–

Method	Purpose					
	Population freedom from infection	**Individual animal freedom from infection prior to movement**	**Contribute to eradication policies**	**Confirmation of clinical cases**	**Prevalence of infection – surveillance**	**Immune status in individual animals or populations post-vaccination**
NA detection by real-time RT-PCR	+++	+++	+++	+++	+++	–
NA detection by ISH	–	–	–	+	–	–
Detection of immune response						
Antibody detection by ELISA	++	++	++	+	++	++
VN	+++	+++	++	+++	+++	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
ELISA = enzyme-linked immunosorbent assay; ~~IHC = immunohistochemistry;~~ NA = nucleic acid; RT-PCR = reverse-transcription polymerase chain reaction; ISH = *in-situ* hybridisation; VN = virus neutralisation.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended.

1. Identification of the agent

There is no designated WOAH Reference Laboratory for BDV, but the reference laboratories for BVDV or CSFV will be able to provide advice[1]. One of the most sensitive proven methods for identifying BDV remains virus isolation. However, a broadly reactive real-time RT-PCR assay (preferably pan-pestivirus reactive) will usually provide higher analytical sensitivity than virus isolation, can be used to test samples that are difficult to manage by virus isolation and can be performed in a few hours. Antigen-detection ELISA and immunohistochemical techniques on tissue sections are also valuable methods for identifying BDV-infected animals.

1.1. Virus isolation

It is essential that laboratories undertaking virus isolation have a guaranteed supply of pestivirus-free susceptible cells and bovine serum, or equivalent, that contain no anti-pestivirus activity and no contaminating virus. It is important that a laboratory quality assurance programme be in place. Chapter 3.4.7 provides detailed methods for virus isolation in either culture tubes or microplates for the isolation of pestiviruses from sheep or goat samples, including serum, whole blood, semen and tissues. The principles and precautions outlined in that chapter for the selection of cell cultures, medium components and reagents are equally relevant to this chapter. Provided proven pan-pestivirus reactive reagents (e.g. monoclonal antibodies [MAbs], primers and probes for real-time RT-PCR) are used for antigen or nucleic acid detection, the principal difference is the selection of appropriate cell cultures.

BD virus can be isolated in a number of primary or secondary ovine cell cultures (e.g. kidney, testes, lung). Ovine cell lines for BDV growth are rare. Semicontinuous cell lines derived from fetal lamb muscle (FLM), whole embryo (Thabti *et al*., 2002) or sheep choroid plexus can be useful, but different lines vary considerably in their susceptibility to the virus. Ovine cells have been used successfully for the isolation and growth of BD viruses and BVDV types 1 and 2 from sheep. In regions where sheep may become infected with BVD viruses from cattle, a virus isolation system using both ovine and bovine cells could be optimal. However, bovine cells have lower sensitivity for the primary

[1] Please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

isolation and growth of some BD viruses, so reliance on bovine cells alone is inadvisable. Details of suitable bovine cell cultures are provided chapter 3.4.7. The precautions outlined in that chapter for the establishment of cells and medium components that are free from contamination with either pestiviruses or antibodies, and measures to ensure that the cells are susceptible to a wide range of local field strains are equally relevant to systems for detection of BDV.

From live animals, serum is the most frequently used sample to be tested for the presence of infectious virus. However, for difficult cases, the most sensitive way to confirm pestivirus viraemia is to wash leukocytes repeatedly (at least three times) in culture medium before co-cultivating them with susceptible cells in either cell culture tubes or microplates. After culture for 5–7 days, the cultures should be frozen and thawed once and an aliquot of diluted culture fluid passaged onto further susceptible cells grown in microplates or on chamber slides to allow antigen detection by immunocytochemistry. Staining for noncytopathic pestiviruses will usually detect virus at the end of the primary passage, but to detect slow-growing viruses in poorly permissive cells two passages are desirable. It is recommended that the culture supernatant used as inoculum for the second passage is diluted approximately 1/100 in new culture medium because some high titred field isolates will replicate poorly if passaged undiluted (i.e. at high multiplicity of infection – moi).

Tissues should be collected from dead animals in virus transport medium. In the laboratory, the tissues are ground to give a 10–20% (w/v) suspension, centrifuged to remove debris, and the supernatant passed through 0.45 µm filters. Spleen, lung, thyroid, thymus, kidney, brain, lymph nodes and gut lesions are the best organs for virus isolation.

Semen can be examined for the presence of BDV, but raw semen is strongly cytotoxic and must be diluted, usually at least 1/10 in culture medium. As the major threat of BDV-infected semen is from PI rams, blood is a more reliable clinical sample than semen for identifying such animals. There are many variations in virus isolation procedures. All should be optimised for maximum sensitivity using a standard reference virus preparation and, whenever possible, recent BDV field isolates. Most of the limitations of virus isolation for the detection of BDV in serum or blood, tissues or semen can be overcome by the use of a proven, sensitive pan-pestivirus reactive real-time RT-PCR. Some laboratories screen samples by real-time RT-PCR and undertake virus isolation on positive samples to collect BDV strains for future reference or research purposes.

For specific technical details of virus isolation procedures, including immunoperoxidase staining, refer to chapter 3.4.7.

1.2. Nucleic acid detection methods

The complete genomic sequences of three BD viruses have been determined and compared with those of other pestiviruses (Becher *et al*., 1998; Ridpath & Bolin, 1997). Phylogenetic analysis shows BD viruses to be more closely related to CSFV than to BVDV (Becher *et al*., 2003; Van Rijn *et al*., 1997; Vilcek & Nettleton, 2006; Vilcek *et al*., 1997). Real-time RT-PCR for diagnosing pestivirus infection is now used widely and a number of formats have been described. Real-time RT-PCR assays have the advantages of being able to detect both infectious virus and residual nucleic acid, the latter being of value for investigating abortions and lamb deaths. Furthermore, the presence of virus-specific antibodies in a sample will have no adverse effect on the sensitivity of the real-time RT-PCR assay. These assays are also useful for screening semen and, when recommended nucleic acid extraction protocols are followed, are less affected by components of the semen compared with virus isolation. Because of the potential for small ruminants to be infected with genetically different strains of BDV or with strains of BVDV, a ~~proven~~ pan-pestivirus reactive real-time RT-PCR with proven high sensitivity should be used. To ensure that the genetic spectrum of BDV strains is sufficiently covered, it may be necessary to apply a broadly reactive BDV specific real time RT-PCR in parallel to maximise diagnostic sensitivity. Suitable protocols for both nucleic acid extraction as well as the real-time RT-PCR are described in chapter 3.4.7. All precautions to minimise laboratory contamination should be followed closely.

After testing samples in a pan-pestivirus reactive assay, samples giving ~~positive results can~~ any level of reactivity should be investigated further by the application of a BDV-specific real-time RT-PCR (Willoughby *et al*., 2006). It is important to note however that different genotypes of BDV may be

circulating in some populations, especially wild ruminants such as chamois and deer, and may be transferred to sheep. An assay that is specific for the detection of BDV should be used with some caution as variants or previously unrecognised genotypes may not be detected, hence the value of initially screening samples with a pan-pestivirus reactive real-time RT-PCR. Nevertheless, there are also situations where a pan-pestivirus reactive real-time RT-PCR may have lower analytical sensitivity. Consequently, in any situation where BDV infection is suspected, the application of several diagnostic methods is recommended. Maternal serology can also play an important role as negative results should exclude the potential involvement of a pestivirus.

1.3. Enzyme-linked immunosorbent assay for antigen detection

ELISAs for the direct detection of pestivirus antigen in blood and tissues of infected animals have proven to be extremely useful for the detection of PI animals and the diagnosis of disease. The first ELISA for pestivirus antigen detection was described for detecting viraemic sheep and was later modified into a double MAb capture ELISA for use in sheep and cattle (Entrican *et al.,* 1994). The test is most commonly employed to identify PI viraemic sheep using washed, detergent-lysed blood leukocytes. The sensitivity is close to that of virus isolation and it is a practical method for screening large numbers of blood samples. As with virus isolation, high levels of colostral antibody can mask persistent viraemia. The ELISA is more effective than virus isolation in the presence of antibody, but may give false-negative results in viraemic lambs younger than 2 months old. The ELISA is usually not sensitive enough to detect acute BDV infections on blood samples. As well as for testing leukocytes, the antigen ELISA can also be used on tissue suspensions, especially spleen, from suspected PI sheep and, as an alternative to immunofluorescence and immunoperoxidase methods, on cell cultures. Several pestivirus ELISA methods have been published but there are at present no commercially available kits that have been fully validated for detecting BDV. Prior to use for regulatory purposes, these kits should be validated in the region where they are to be used to ensure that a wide range of field strains of BDV can be detected and that they are suitable for the sample types to be tested.

1.4. Immunohistochemistry

Viral antigen demonstration is possible in most of the tissues of PI animals (Braun *et al*., 2002; Thur *et al*., 1997) although this is not a method that is routinely used for diagnostic purposes. This should be done on acetone-fixed frozen tissue sections (cryostat sections) or paraffin wax embedded samples using appropriate antibodies. Pan-pestivirus reactive antibodies with NS2-3 specificity are suitable. Tissues with a high amount of viral antigen are brain, thyroid gland, lung and oral mucosa. Skin biopsies have been shown to be useful for *in-vivo* diagnosis of persistent BDV infection.

2. Serological tests

Antibody to BDV is usually detected in sheep sera using VN or an ELISA. The less sensitive agar gel immunodiffusion test is not recommended. Control positive and negative reference sera must be included in every test. These should give results within predetermined limits for the test to be considered valid. Single sera can be tested to determine the prevalence of BDV in a flock, region or country. For diagnosis, however, acute and convalescent sera are the best samples for confirming acute BDV infection. Repeat sera from one animal should always be tested alongside each other on the same plate to provide a reliable comparison of titres.

2.1. Virus neutralisation test

Due to antigenic diversity among pestiviruses the choice of test virus is difficult (Dekker *et al*., 1995; Nettleton *et al*., 1998). No single strain of BDV is ideal. A local strain that gives the highest antibody titre with a range of positive sheep sera should be used.

Because there are few cytopathogenic strains of BDV available, to achieve optimal analytical sensitivity, it is more usual to employ a representative local non-cytopathogenic strain and read the assay after immunoperoxidase staining of the cells. Proven highly sensitive, pestivirus-free sheep cells such as lamb testis or kidney cells are suitable and can be maintained as cryogenically frozen stocks for use over long periods of time. The precautions outlined for selection of pestivirus-free

medium components are equally applicable to reagents to be used in VN tests. A recommended procedure follows.

2.1.1. Test procedure

i) The test sera are heat-inactivated for 30 minutes at 56°C.

ii) From a starting dilution of 1/4, serial twofold dilutions of the test sera are made in a cell-culture grade flat-bottomed 96-well microtitre plate, using cell culture medium as diluent. For each sample, three or four wells are used at each dilution depending on the degree of precision required. Also, for each sample and at each serum dilution, one well is left without virus to monitor for evidence of sample toxicity that could mimic viral cytopathology or interfere with virus replication. Control positive and negative sera should also be included in each batch of tests.

iii) An equal volume (e.g. 50 µl) of a stock of BDV containing 100 $TCID_{50}$ (50% tissue culture infective dose) is added to each well. A back titration of virus stock is also done in some spare wells to check the potency of the virus (acceptance limits 30–~~80~~–300 $TCID_{50}$).

iv) The plate is incubated for 1 hour at 37°C.

v) A flask of suitable cells (e.g. ovine testis or kidney cells) is trypsinised and the cell concentration is adjusted to 2×10^5/ml. 100 µl of the cell suspension is added to each well of the microtitre plate.

vi) The plate is incubated at 37°C for 4–5 days, either in a 5% CO_2 atmosphere or with the plate sealed.

vii) The wells are examined microscopically to ensure that there is no evidence of toxicity or cytopathic effect (CPE), then fixed and stained by immunoperoxidase staining using an appropriate MAb. The VN titre for each serum is the dilution at which the virus is neutralised in 50% of the wells. This can be calculated by the Spearman–Kärber or Reed Muench methods. A seronegative animal will show no neutralisation at the lowest dilution of serum (i.e. 1/4), equivalent to a final dilution of 1/8. For accurate comparison of antibody titres, and particularly to demonstrate significant (more than fourfold) changes in titre, samples should be tested in parallel in the same test.

viii) Occasionally there may be a need to determine whether antibody in a flock is against a virus belonging to a particular *Pestivirus* serogroup. A differential VN test can be used in which sera are titrated out against representative viruses from each of the four *Pestivirus* groups, i.e. BDV, BVDV types 1 and 2, and CSFV. Maximum titre will identify the infecting serotype and the spectrum of cross-reactivity with the other serotypes will also be revealed.

2.2. Enzyme-linked immunosorbent assay

An MAb-capture ELISA for measuring BDV antibodies has been described. Two pan-pestivirus MAbs that detect different epitopes on the immunodominant nonstructural protein NS 2/3 are used to capture detergent-lysed cell-culture grown antigen. The results correlate qualitatively with the VN test (Fenton *et al*., 1991).

2.2.1. Antigen preparation

Use eight 225 cm^2 flasks of newly confluent FLM cells; four flasks will be controls and four will be infected. Wash the flasks and infect four with a 0.01–0.1 m.o.i. of Moredun cytopathic BDV. Allow the virus to adsorb for 2 hours at 37°C. Add maintenance media containing 2% FBS (free from BDV antibody), and incubate cultures for 4–5 days until CPE is obvious. Pool four control flask supernatants and separately pool four infected flask supernatants. Centrifuge at 3000 ***g*** for 15 minutes to pellet cells. Discard the supernatants. Retain the cell pellets. Wash the flasks with 50 ml of PBS and repeat the centrifugation step as above. Pool all the control cell pellets in 8 ml PBS containing 1% Nonidet P40 and return 2 ml to each control flask to lyse the remaining attached cells. Repeat for infected cells. Keep the flasks at 4°C for at least 2 hours agitating the small volume of fluid on the cells vigorously every 30 minutes to ensure

total cell detachment. Centrifuge the control and infected antigen at 12,000 ***g*** for 5 minutes to remove the cell debris. Supernatant antigens are stored at –70°C in small aliquots.

2.2.2. Test procedure

i) The two MAbs are diluted to a predetermined dilution in 0.05 M bicarbonate buffer, pH 9.6. All wells of a suitable ELISA-grade microtitre plate (e.g. Nunc maxisorb, Greiner 129b) are coated overnight at 4°C.

ii) After washing three times in PBST, a blocking solution of PBST containing 10% horse serum (PBSTH) is added to all wells, which are incubated at 37°C for 1 hour.

iii) The antigen is diluted to a predetermined dilution in PBSTH and alternate rows of wells are coated with virus and control antigens for 1 hour at 37°C. The plates are then washed three times in PBST before addition of test sera.

iv) Test sera are diluted 1/50 in PBSTH and added to duplicate virus and duplicate control wells for 1 hour at 37°C. The plates are then washed three times in PBST.

v) Anti-ovine IgG peroxidase conjugate is diluted to a predetermined dilution in PBSTH and added to all wells for 1 hour at 37°C. The plates are washed three times in PBST.

vi) A suitable activated enzyme substrate/chromogen, such as ortho-phenylene diamine (OPD) or tetramethyl benzidine (TMB), is added). After colour development, the reaction is stopped with sulphuric acid and the absorbance read on an ELISA plate reader. The mean value of the two control wells is subtracted from the mean value of the two virus wells to give the corrected absorbance for each serum. Results are expressed as corrected absorbance with reference to the corrected absorbance of known positive and negative sera. Alternatively, ELISA titres can be extrapolated from a standard curve of a dilution series of a known positive reference serum.

If antigens of sufficient potency can be produced the MAb capture stage can be omitted. In this case alternate rows of wells are coated with virus and control antigen diluted to a predetermined dilution in 0.05 M bicarbonate buffer, pH 9.6, overnight at +4°C. The plates are washed and blocked as in step ii above. After washing, diluted test sera are added and the test proceeds from step iv as above.

C. REQUIREMENTS FOR VACCINES

1. Background

To be useful, a BDV vaccine should be effective when administered to female sheep before breeding to prevent transplacental infection. Experimental and commercial inactivated whole virus BDV vaccines have been produced in Europe (Brun *et al*., 1993; Vantsis *et al*., 1980). Unlike vaccines for BVDV, there is limited demand for vaccines against BDV and those produced have only been inactivated products. No live attenuated or recombinant subunit vaccines for BDV have been produced commercially.

Pestivirus contaminants of modified live virus vaccines have been found to be a cause of serious disease following their use in pigs, cattle, sheep and goats. Contaminated vaccines have included those used for the control of Aujesky's disease, CSF, rotavirus, coronavirus, rinderpest, sheep pox and contagious pustular dermatitis. The insidious ability of pestiviruses to cross the placenta, and thus establish PI animals, gives them the potential to contaminate vaccines through cells, serum used as medium supplement, or seed stock virus. As nearly all isolates of pestiviruses are noncytopathic, they will remain undetected unless specific tests are carried out. Although such contamination should be less likely to be a problem with an inactivated vaccine, nevertheless steps should be taken to ensure that materials used in production are not contaminated.

1.1. Characteristics of a target product profile

Traditionally, pestivirus vaccines fall into two classes: modified live or inactivated virus vaccines. The essential requirement for both types is to ~~afford~~ provide a high level of fetal infection. Only inactivated vaccines have been produced for BDV. Properly formulated inactivated vaccines are very safe to use but, to obtain satisfactory levels of immunity, they usually require booster vaccinations, which may be inconvenient. Because of the propensity for antigenic variability, the vaccine should contain strains of BDV that are closely matched to viruses found in the area in which they are used. This may present particular challenges with BDV in regions where several antigenic types have been found. Due to the need to customise vaccines for the most commonly encountered strains within a country or region, it is not feasible to produce a vaccine antigen bank that can be drawn upon globally.

Guidance for the production of veterinary vaccines is given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

An ideal vaccine should contain a strain or strains of virus that give protection against all sheep pestiviruses. This may be challenging however, because of the range of pestiviruses with which sheep can be infected. There is considerable antigenic variation across these viruses – both between viruses that have been classified in the BDV genogroup as well as between viruses in the BVDV1 and BVDV2 genotypes (Becher *et al*., 2003; Vilcek & Nettleton, 2006; Wensvoort *et al*., 1989). Infection of sheep with the putative BVDV-3 genotype has also been described (Decaro *et al.,* 2012). It is likely that the antigenic composition of a vaccine will vary from region to region to provide an adequate antigenic match with dominant virus strains. Cross-neutralisation studies are required to establish optimal combinations. Nevertheless, it would appear that any BDV vaccine should contain at least a representative of the BDV and BVDV (type 1) groups. Characterisation of the biologically cloned vaccine viruses should include typing with MAbs and genotyping (Paton *et al*., 1995).

2.1.1. Quality criteria (sterility, purity, freedom from extraneous agents)

It is crucial to ensure that all materials used in the preparation of the bulk antigens have been extensively screened to ensure freedom from extraneous agents. This should include master and working seeds, the cell cultures and all medium supplements such as bovine serum. Some bovine viruses and particularly BVDV can readily infect small ruminants such as sheep. Therefore, it is particularly important to ensure that any serum used that is of bovine origin is free of both adventitious BVDV and antibodies against BVDV strains because low levels of either virus or antibody can mask the presence of the other. Materials and vaccine seeds should be tested for sterility and freedom from contamination with other agents, especially viruses as described in the chapter 1.1.8 and Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*.

If a vaccine passes basic tests, the efficacy of vaccination should ultimately be measured by the capacity to prevent transplacental transmission. Effective challenge of vaccinated pregnant ewes at 50–60 days gestation has been achieved by intranasal installation of virus or by mixing with PI sheep (Brun *et al.,* 1993). Usually this reliably produces persistently viraemic offspring in non-immune ewes. In regions where multiple genotypes of BDV viruses are commonly encountered, efficacy in protecting against multiple strains should be measured.

2.2. Method of manufacture

2.2.1. Procedure

Inactivated vaccines have been prepared using conventional laboratory techniques with stationary or rolled cell cultures. Inactivants have included formalin and beta-propriolactone. Adjuvants have included aluminium hydroxide and oil (Brun *et al*., 1993; Vantsis *et al*., 1980). Optimal yields depend on the cell type and isolate used. A commercial BDV vaccine containing

two strains of virus has been prepared on ovine cell lines (Brun *et al*., 1993). Cells must be produced according to a seed-lot system from a master cell seed (MCS) that has been shown to be free from all contaminating microorganisms. Vaccine should only be produced in cells fewer than 20 passages from the MCS. Control cells from every passage should be checked for pestivirus contamination. Standard procedures may be used, with the expectation for harvesting noncytopathic virus on days 4–7 after inoculation of cultures. The optimal yield of infectious virus will depend on several factors, including the cell culture, isolate used and the initial seeding rate of virus. These factors should be taken into consideration and virus replication kinetics investigated to establish the optimal conditions for large-scale virus production. Whether a live or inactivated vaccine, the essential aim will be to produce a high-titred virus stock. This bulk antigen preparation can subsequently be prepared according to the type of vaccine being considered.

2.2.2. Requirements for ingredients

BDV vaccines have usually been grown in cell cultures of ovine origin that are frequently supplemented with medium components of animal origin. The material of greatest concern is bovine serum due to the potential for contamination with BVD viruses and antibodies to these viruses. These adventitious contaminants not only affect the efficiency of production but also may mask the presence of low levels of infectious BVDV that may have undesirable characteristics. In addition to the virus seeds, all materials should be tested for sterility and freedom from contamination with other agents, especially viruses as described in chapters 1.1.8 and 1.1.9. Furthermore, materials of bovine or ovine origin should originate from a country with negligible risk for transmissible spongiform encephalopathies (see chapter 1.1.9).

2.2.3. In-process controls

In-process controls are part of the manufacturing process. Cultures should be inspected regularly to ensure that they remain free from gross bacterial contamination, and to monitor the health of the cells and the development or absence of CPE, as appropriate. While the basic requirement for efficacy is the capacity to induce an acceptable neutralising antibody response, during production, target concentrations of antigen required to achieve an acceptable response may be monitored indirectly by assessment of the quantity of infectious virus or antigen mass that is produced. Rapid diagnostic assays such as the ELISA are useful for monitoring BVDV antigen production. Alternatively, the quality of a batch of antigen may be determined by titration of the quantity of infectious virus present, although this may underestimate the quantity of antigen. For inactivated vaccines, infectivity is evaluated before inactivation. For inactivated vaccines the inactivation kinetics should be established so that a suitable safety margin can be determined and incorporated into the routine production processes. At the end of production, *in-vitro* cell culture assays should be undertaken to confirm that inactivation has been complete. These innocuity tests should include a sufficient number of passages and volume of inoculum to ensure that very low levels of infectious virus would be detected if present.

2.2.4. Final product batch tests

i) Sterility

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

ii) Identity

Identity tests should demonstrate that no other strain of BDV is present when several strains are propagated in a facility producing multivalent vaccines.

iii) Safety

Samples from inactivated vaccines should be tested rigorously for viable virus. Samples of the product should be passaged for a minimum of three passages in sensitive cell cultures to ensure absence of live BDV. This *in-vitro* monitoring can be augmented by injecting two BDV-seronegative sheep with 20 doses of unformulated antigen as part of

a standard safety test. Presence of live virus will result in the development of a more convincing serological response than will occur with inactivated virus alone. The sheep sera can also be examined for antibody to other prescribed agents.

Safety tests shall also consist of detecting any abnormal local or systemic adverse reactions to the vaccine by all vaccination route(s). Batch-to-batch safety tests are required unless safety of the product is demonstrated and approved in the registration dossier and production is consistent with that described in chapter 1.1.8. Vaccines must either be demonstrated to be safe in pregnant sheep (i.e. no transmission to the fetus), or should be licensed with a warning not to use them in pregnant animals.

iv) Batch potency

Vaccine potency is best tested in seronegative sheep in which the development and level of antibody is measured. BVD vaccines must be demonstrated to produce adequate immune responses when used in their final formulation according to the manufacturer's published instructions. The minimum quantity of infectious virus or antigen required to produce an acceptable immune response should be determined. An indirect measure of potency is given by the level of virus infectivity prior to inactivation. *In-vitro* assays should be used to monitor individual batches during production. The antigen content following inactivation can be assayed by MAb-capture ELISA and related to the results of established *in-vivo* potency results. It should be demonstrated that the lowest recommended dose of vaccine can prevent transplacental transmission of BDV in pregnant sheep.

2.3. Requirements for authorisation/registration/licensing

2.3.1. Manufacturing process

For registration of a vaccine, all relevant details concerning manufacture of the vaccine and quality control testing should be submitted to the relevant authorities. Unless otherwise specified by the authorities, information should be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

There is no standard method for the manufacture of a BDV vaccine, but conventional laboratory techniques with stationary, rolled or suspension (micro-carriers) cell cultures may be used. Inactivated vaccines can be prepared by conventional methods, such as binary ethylenimine, formalin or beta-propiolactone inactivation (Park & Bolin, 1987). A variety of adjuvants may be used.

2.3.2. Safety requirements

In-vivo tests should be undertaken using repeat doses (taking into account the maximum number of doses for primary vaccination and, if appropriate, the first revaccination/booster vaccination) and contain the maximum permitted antigen load and, depending on the formulation of the vaccine, the maximum number of vaccine strains.

i) Target and non-target animal safety

The safety of the final product formulation of inactivated vaccines should be assessed in susceptible young sheep that are free of maternally derived antibodies and in pregnant ewes. They should be checked for any local reactions following administration, and, in pregnant ewes, for any effects on the unborn lamb.

ii) Reversion-to-virulence for attenuated/live vaccines and environmental considerations

In the event that a live virus vaccine was developed for BDV, virus seeds that have been passaged at least up to and preferably beyond the passage limit specified for the seed should be inoculated into young lambs to confirm that there is no evidence of disease. If a live attenuated vaccine has been registered for use in pregnant animals, reversion to

virulence tests should also include pregnant animals. Live attenuated vaccines should not be transmissible to unvaccinated 'in-contact' animals.

iii) Precautions (hazards)

BDV is not considered to be a human health hazard. Standard good microbiological practice should be adequate for handling the virus in the laboratory. While the inactivated virus in a vaccine should be identified as harmless for people administering the product, adjuvants included in the vaccine may cause injury to people. Manufacturers should provide adequate warnings that medical advice should be sought in the case of self-injection (including for adjuvants, oil-emulsion vaccine, preservatives, etc.) with warnings included on the product label/leaflet so that the vaccinator is aware of any danger.

2.3.3. Efficacy requirements

The potency of the vaccine should be determined by inoculation into seronegative and virus negative lambs, followed by monitoring of the antibody response. Antigen content can be assayed by infectivity titration prior to inactivation and subsequently by ELISA and adjusted as required to a standard level for the particular vaccine. Standardised assay protocols applicable to all vaccines do not exist. Live vaccine batches may be assayed by infectivity titration. Each production batch of vaccine should undergo potency and safety testing as batch release criteria. BVD vaccines must be demonstrated to produce adequate immune responses, as outlined above, when used in their final formulation according to the manufacturer's published instructions.

2.3.4. Vaccines permitting a DIVA strategy (detection of infection in vaccinated animals)

To date, there are no commercially available vaccines for BDV that support use of a true DIVA strategy.

2.3.5. Duration of immunity

Inactivated vaccines are unlikely to provide sustained levels of immunity and it is likely that after an initial course of two or three injections annual booster doses may be required. Insufficient information is available to determine any correlation between vaccinal antibody titres in the dam and fetal protection. As there are likely to be different commercial formulations and these involve a range of adjuvants, there are likely to be different periods of efficacy. Consequently, duration of immunity data must be generated separately for each commercially available product by undertaking challenge tests at the end of the period for which immunity has been claimed.

2.3.6. Stability

There are no accepted guidelines for the stability of BDV vaccines, but it can be assumed that an inactivated virus vaccine should remain potent for at least 1 year if kept at 4°C and probably longer. Lower temperatures could prolong shelf life but adjuvants in a killed vaccine may preclude this. Bulk antigens that have not been formulated into finished vaccine can be reliably stored frozen at low temperatures, but the antigen quality should be monitored with *in-vitro* assays prior to incorporation into a batch of vaccine.

REFERENCES

BARLOW R.M. & PATTERSON D.S.P. (1982). Border disease of sheep: a virus-induced teratogenic disorder. *Adv. Vet. Med.* (*Suppl. J. Vet. Med.*), **36**, 1–87.

BECHER P., AVALOS-RAMIREZ R., ORLICH M., CEDILLO ROSALES S., KONIG, M., SCHWEIZER M., STALDER H., SCHIRRMEIR H & THIEL H.-J. (2003). Genetic and antigenic characterisation of novel pestivirus genotypes; Implications for classification. *Virology,* **311**, 96–104.

BECHER P., ORLICH M. & THIEL H.-J. (1998). Complete genomic sequence of border disease virus a pestivirus from sheep. *J. Virol.*, **72**, 5165–5173.

BRAUN U., HILBE M., EHRENSPERGER F., SALIS F., ALTHER P., STRASSER M., STALDER H.P. & PETERHANS E. (2002). Border Disease in einem Schafbetrieb. *Schweiz. Arch. Tierheilk*., **144**, 419–426.

BRUN A., LACOSTE F., REYNAUD G., KATO F. & SAINT-MARC B. (1993). Evaluation of the potency of an inactivated vaccine against border disease pestivirus infection in sheep. *In:* Proceedings of the Second Symposium on Pestiviruses, Edwards S., ed. Fondation Marcel Merieux, Annecy, France, 1–3 October 1992, 257–259

CARLSSON U. (1991). Border disease in sheep caused by transmission of virus from cattle persistently infected with bovine virus diarrhoea virus. *Vet. Rec*., **128**, 145–147.

CHAPPUIS G., BRUN A., KATO F., DAUVERGNE M., REYNAUD G. & DURET C. (1986). Etudes serologiques et immunologiques realisees a la suite de l'isolement d'un pestivirus dans un foyer ovina chez des moutons de L'Aveyron. *In:* Pestiviroses des Ovins et des Bovins, Espinasse J. & Savey M. eds. Ste Françoise de Buiatrie, Paris, France, **55**, 66.

DECARO N., MARI V., LUCENTE M., SCIARRETTA R., MORENO A., ARMENISE C., LOSURDO M., CAMERO M., LORUSSO E., CORDIOLI P., & BUONAVOGLIA C. (2012). Experimental infection of cattle, sheep and pigs with 'Hobi'-like pestivirus. *Vet. Microbiol*., **155**, 165–171.

DEKKER A., WENSVOORT G. & TERPSTRA C. (1995). Six antigenic groups within the genus pestivirus as identified by cross-neutralisation assays. *Vet. Microbiol*., **47**, 317–329.

ENTRICAN G., DAND A. & NETTLETON P.F. (1994). A double monoclonal-antibody ELISA for detecting pestivirus antigen in the blood of viraemic cattle and sheep. *Vet. Microbiol*., **43**, 65–74.

FENTON A., SINCLAIR J.A., ENTRICAN G., HERRING J.A. & NETTLETON P.F. (1991). A monoclonal antibody capture ELISA to detect antibody to border disease virus in sheep sera. *Vet. Microbiol*., **28**, 327–333.

~~INTERNATIONAL COMMITTEE ON TAXONOMY OF VIRUSES (2016). Virus Taxonomy 2015 release. http://www.ictvonline.org/virusTaxonomy.asp~~

NETTLETON P.F., GILMOUR J.S., HERRING J.A. & SINCLAIR J.A. (1992). The production and survival of lambs persistently infected with border disease virus. *Comp. Immunol. Microbiol. infect. Dis*., **15**, 179–188.

NETTLETON P.F., GILRAY J.A., RUSSO P. & DLISSI E. (1998). Border disease of sheep and goats *Vet. Res.,* **29**, 327–340.

OGUZOGLU T.C., FLOEGEL-NIESMANN G., FREY H.R. & MOENNIG V. (2001). Differential diagnosis of classical swine fever and border disease: seroepidemiological investigation of a pestivirus infection on a mixed sheep and swine farm. *Dtsch Tierarztl. Wochenschr.*, **108**, 210–213.

PARK B.K. & BOLIN S.R. (1987). Molecular changes of bovine viral diarrhea virus polypeptides treated with binary ethylenimine, beta-propiolactone and formalin. *Res. Rep. Rural Dev. Admin.* (*L&V*) *Korea*, **29**, 99–103.

PATON D.J., SANDS J.J., LOWINGS J.P., SMITH J.E., IBATA G. & EDWARDS S. (1995). A proposed division of the pestivirus genus into subgroups using monoclonal antibodies, supported by cross-neutralization assays and genetic sequencing. *Vet. Res*., **26**, 92–109.

POSTLER T.S., BEER M., BLITVICH B.J., BUKH J., DE LAMBALLERIE X., DREXLER J.F., IMRIE A., KAPOOR A., KARGANOVA G.G., LEMEY P., LOHMANN V., SIMMONDS P., SMITH D.B., STAPLETON J.T. & KUHN J.H. (2023). Renaming of the genus Flavivirus to Orthoflavivirus and extension of binomial species names within the family Flaviviridae. *Arch. Virol.*, **168**, DOI: 10.1007/s00705-023-05835-1

RIDPATH J.F. & BOLIN S.R. (1997). Comparison of the complete genomic sequence of the border disease virus, BD31, to other pestiviruses. *Virus Res.,* **50**, 237–243.

RIGHI C., PETRINI S., PIERINI I., GIAMMARIOLI M. & DE MIA G.M. (2021). Global Distribution and Genetic Heterogeneity of Border Disease Virus. *Viruses*, **13**, 950. doi: 10.3390/v13060950. PMID: 34064016; PMCID: PMC8223970.

THABTI F., FRONZAROLI L., DLISSI E., GUIBERT J.M., HAMMAMI S., PEPIN M. & RUSSO P. (2002). Experimental model of border disease virus infection in lambs: comparative pathogenicity of pestiviruses isolated in France and Tunisia. *Vet. Res.,* **33**, 35–45.

THUR B., HILBE M., STRASSER M. & EHRENSPERGER F. (1997). Immunohistochemical diagnosis of pestivirus infection associated with bovine and ovine abortion and perinatal death. *Am. J. Vet. Res.,* **58**, 1371–1375.

VALDAZO-GONZALEZ B., ALVAREZ-MARTINEZ M. & SANDVIK T. (2007). Genetic and antigenic typing of border Disease virus isolates in sheep from the Iberian peninsula. *Vet. J*., **174**, 316–324.

VAN RIJN P.A., VAN GENNIP H.G.P., LEENCLERSE C.H., BRUSCHKE C.J.M., PATON D.J., MOORMANN R.J.M. & VAN OIRSCHOT J.T. (1997). Subdivision of the pestivirus genus based on envelope glycoprotein E2 *Virology*, **237**, 337–348.

VANTSIS J.T., BARLOW R.M., FRASER J. & MOULD D.L. (1976). Experiments in border disease VIII. Propagation and properties of a cytopathic virus. *J. Comp. Pathol*., **86**, 111–120.

VANTSIS J.T., RENNIE J.C., GARDINER A.C., WELLS P.W., BARLOW R.M. & MARTIN W.B. (1980). Immunisation against Border disease. *J. Comp. Path*., **90**, 349–354.

VILCEK S. & NETTLETON P.F. (2006). Pestiviruses in wild animals *Vet. Microbiol.,* **116**, 1–12.

VILCEK S., NETTLETON P.F., PATON D.J. & BELAK S. (1997). Molecular characterization of ovine pestiviruses. *J. Gen. Virol.,* **78**, 725–735.

WENSVOORT G. & TERPSTRA C. (1988). Bovine viral diarrhoea virus infection in piglets born to sows vaccinated against swine fever with contaminated virus. *Res. Vet. Sci.,* **45**, 143–148.

WENSVOORT G., TERPSTRA C. & DE KLUYVER E.P. (1989). Characterisation of porcine and some ruminant pestiviruses by cross-neutralisation. *Vet. Microbiol.*, **20**, 291–306.

WILLOUGHBY K., VALDAZO-GONZALEZ, B., MALEY M., GILRAY J. & NETTLETON P.F. (2006). Development of a real time RT-PCR to detect and type ovine pestiviruses. *J. Virol. Methods,* **132,** 187–194.

*
* *

NB: At the time of publication (2017) there were no WOAH Reference Laboratories for border disease (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3http://www.oie.int/).

NB: FIRST ADOPTED IN 1996. MOST RECENT UPDATES ADOPTED IN 2017.

Annex 15. Chapter 3.8.12. 'Sheep pox and goat pox'

CHAPTER 3.8.12.

SHEEP POX AND GOAT POX

SUMMARY

Sheep pox and goat pox are contagious, viral diseases of sheep and goats characterised by fever, generalised papules or nodules, vesicles (rarely), internal lesions (particularly in the lungs), and death. Both diseases are caused by strains of capripoxvirus, all of which can infect sheep and goats. Although most of the strains examined cause more severe clinical disease in either sheep or goats, some strains have been isolated that are equally pathogenic in both species.

Sheeppox virus (SPPV) and goatpox virus (GTPV) are the causative agents of sheep pox and goat pox, and with lumpy skin disease virus (LSDV) make up the genus Capripoxvirus *in the family* Poxviridae*. Sheep pox and goat pox are endemic in Africa north of the Equator, the Middle East and Asia, while some parts of Europe have experienced outbreaks recently. See WAHIS (https://wahis.woah.org/#/home) for recent information on distribution at the country level. ~~Countries that reported outbreaks of the disease between 2010 and 2015 include Bulgaria, Chinese Taipei, Israel, Kazakhstan, Kyrgyzstan, Mongolia, Morocco, Greece and Russia, with Greece, Israel and Russia having experienced recurring incidences.~~*

***Identification of the agent:** Laboratory confirmation of capripoxvirus is most rapid using the polymerase chain reaction (PCR) method in combination with a clinical history consistent with generalised capripoxvirus infection. Isolation of the virus is possible as capripoxviruses will grow on tissue culture of ovine, caprine or bovine origin, although field isolates may require up to 14 days to grow or require one or more additional tissue culture passage(s). The virus causes intracytoplasmic inclusions that can be clearly seen using haematoxylin and eosin staining. The antigen can also be detected in tissue culture using specific sera and immunoperoxidase or immunofluorescence techniques. Capripoxvirus antigen and inclusion bodies may be seen in stained cryostat or paraffin sections of biopsy or post-mortem lesion material.*

~~An antigen-detection enzyme-linked immunosorbent assay (ELISA) using a polyclonal detection serum raised against a recombinant immunodominant antigen of capripoxvirus has been developed.~~

***Serological tests:** The virus neutralisation test is the most specific serological test. The indirect immunofluorescence test is less specific due to cross-reactions with antibody to other poxviruses. Western blotting using the reaction between the P32 antigen of capripoxvirus with test sera is both sensitive and specific, but is expensive and difficult to carry out. An enzyme-linked immunosorbent assay (ELISA) has been developed and validated to detect antibodies to capripoxviruses, however it cannot differentiate between SPPV, GTPV and LSDV.*

~~The use of this antigen, or other appropriate antigens expressed by a suitable vector, in an ELISA offers the prospect of an acceptable and standardised serological test in the future.~~

Requirements for vaccines: *Live and inactivated vaccines have been used for the control of capripoxviruses. All strains of capripoxvirus so far examined share a major neutralisation site and some will cross protect. Inactivated vaccines give, at best, only short-term immunity.*

A. INTRODUCTION

The *Capripoxvirus* genus, in the family *Poxviridae,* consists of three species – lumpy skin disease virus (LSDV), which causes disease in cattle only (see Chapter 3.4.12), and sheeppox virus (SPPV) and goatpox virus (GTPPV), which cause sheep pox and goat pox, respectively. Sheep pox and goat pox are characterised by disseminated cutaneous nodules and up to 100% mortality in fully ~~susceptible breeds~~ naïve of sheep and goats. In indigenous animals, generalised disease and mortality are less common, although they are seen where disease has been absent from an area or village for a period of time, when intensive husbandry methods are introduced, or in association with other disease agents, such as peste des petits ruminants virus or foot and mouth disease virus. Sheep pox and goat pox are major constraints to the introduction of exotic breeds of sheep and goats to endemic areas, and to the development of intensive livestock production.

Strains of SPPV and GTPV can pass between sheep and goats, although most cause more severe clinical disease in ~~only one~~ their homologous host species. SPPV and GTPV are transboundary diseases that regularly spread into adjacent, non-endemic areas. Sheep pox and goat pox are endemic in Africa north of the Equator and parts of the Middle East and Asia (see WAHIS for most up-to-date information on distribution: https://wahis.woah.org/#/home). Outbreaks have been reported in non-endemic countries of Asia, Europe and the Middle East.

The incubation period of sheep pox and goat pox is between 8 and 13 days following contact between infected and susceptible animals. It may be as short as 4 days following experimental infection by intradermal inoculation ~~or mechanical transmission by insects~~. Some breeds of European sheep, such as Soay, may die of acute infection before the development of skin lesions. In other breeds there is an initial rise in rectal temperature to above 40°C, followed in 2–5 days by the development of, at first, macules – small circumscribed areas of hyperaemia, which are most obvious on unpigmented skin – and then of papules – hard swellings of between 0.5 and 1 cm in diameter – which may cover the body or be restricted to the groin, axilla and perineum. Papules may be covered by fluid-filled vesicles, but this is rare. Some researchers have distinguished between a vesicular and nodular form of sheep pox and goat pox (Zro *et al*., 2014b).

Within 24 hours of the appearance of generalised papules, affected animals develop rhinitis, conjunctivitis and enlargement of all the superficial lymph nodes, in particular the prescapular lymph nodes. Papules on the eyelids cause blepharitis of varying severity. As the papules on the mucous membranes of the eyes and nose ulcerate, so the discharge becomes mucopurulent, and the mucosae of the mouth, anus, and prepuce or vagina become necrotic. Breathing may become laboured and noisy due to pressure on the upper respiratory tract from the swollen retropharyngeal lymph nodes, due to the developing lung lesions.

If the affected animal does not die in this acute phase of the disease, the papules start to become necrotic from ischaemic necrosis following thrombi formation in the blood vessels at the base of the papule. In the following 5–10 days the papules form scabs, which persist for up to 6 weeks, leaving small scars. The skin lesions are susceptible to fly strike, and secondary pneumonia is common. Anorexia is not usual unless the mouth lesions physically interfere with feeding. Abortion is rare.

On post-mortem examination of the acutely infected animal, the skin lesions are often less obvious than on the live animal. The mucous membranes appear necrotic and all the body lymph nodes are enlarged and oedematous. Papules, which may be ulcerated, can usually be found on the abomasal mucosa, and sometimes on the wall of the rumen and large intestine, on the tongue, hard and soft palate, trachea and oesophagus. Pale areas of approximately 2 cm in diameter may occasionally be seen on the surface of the kidney and liver, and have been reported to be present in the testicles. Numerous hard lesions of up to 5 cm in diameter are commonly observed throughout the lungs, but particularly in the diaphragmatic lobes.

The clinical signs and post-mortem lesions vary considerably with breed of host and strain of capripoxvirus. Indigenous breeds are less susceptible and frequently show only a few lesions, which could be confused with insect bites or contagious pustular dermatitis. However, lambs that have lost their maternally derived immunity,

animals that have been kept isolated and animals brought into endemic areas from isolated villages, particularly if they have been subjected to the stress of moving long distances and mixing with other sheep and goats, and their pathogens, can often be seen with generalised and sometimes fatal capripoxvirus infections. Invariably there is high mortality in unprotected imported breeds of sheep and goats following capripoxvirus infection. Surviving animals clear the infection, as there is no evidence of persistently infected animals. Capripoxvirus is not infectious to humans. Capripoxvirus is inactivated at 56°C for 2 hours or 65°C for 30 minutes. The virus survives between pH 6.6–8.6. It is susceptible to highly alkaline or acid pH. The virus is sensitive to various chemicals: sodium dodecyl sulphate, ether 20%, chloroform, formalin 1%, sodium 2%, iodine compounds, Virkon 2%, quaternary ammonium (0.5%), and phenol 2% for 15 minutes.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for diagnosis of sheep pox and goat pox and their purpose

Method	Purpose					
	Population freedom from infection	**Individual animal freedom from infection prior to movement**	**Contribute to eradication policies**	**Confirmation of clinical cases**	**Prevalence of infection – surveillance**	**Immune status in individual animals or populations post-vaccination**
Identification of the agent[(a)]						
Virus isolation	+	++	+	+++	+	–
~~**Antigen detection**~~	~~++~~	~~++~~	~~++~~	~~++~~	~~++~~	~~–~~
IFAT	±	±	±	++	±	±
IHC	±	±	±	++	±	±
PCR	++	+++	++	+++	++	–
Detection of immune response						
VNT	++	++	++	++	++	++
IFAT	+	+	+	+	+	+
ELISA	++	++	++	++	++	++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
IFAT = indirect fluorescent antibody test; IHC = ; immunohistochemistry; PCR = polymerase chain reaction;
VNT = virus neutralisation; ELISA = enzyme-linked immunosorbent assay.
[(a)]~~A combination of agent identification methods applied on the same clinical sample is recommended.~~

1. Identification of the agent

1.1. Specimen collection and submission

Material for virus isolation ~~and antigen detection~~ should be collected by biopsy or at post-mortem from skin papules, lung lesions or lymph nodes. Samples for virus isolation ~~and antigen detection enzyme-linked immunosorbent assay (ELISA)~~ should be collected within the first week of the occurrence of clinical signs, before the development of neutralising antibodies. Samples for genome detection by polymerase chain reaction (PCR) may be collected before or after the development of neutralising antibody responses. In addition to epithelial lesions, nasal and buccal swabs can be

collected because the virus will be present in nasal and saliva discharges. Buffy coat from blood collected into EDTA (ethylene diamine tetra-acetic acid) during the viraemic stage of capripoxvirus infection (before generalisation of lesions or within 4 days of generalisation), can also be used for virus isolation.

Samples for histology should include tissue from the surrounding area and should be placed immediately following collection into ten times the sample volume of 10% formalin or neutral buffered 10% formal saline. ~~Tissues in formalin have no special transportation requirements.~~

Blood samples, for virus isolation from the buffy coat, should be collected in tubes containing anticoagulant, placed immediately on ice and processed as soon as possible. In practice, the blood samples may be kept at 4°C for up to 2 days prior to processing, but should not be frozen or kept at ambient temperatures. Tissues and dry scabs for virus isolation, ~~antigen detection~~ and genome detection should preferably be kept at 4°C, on ice or at –20°C. If it is necessary to transport samples over long distances without refrigeration, the medium should contain 10% glycerol; the samples should be of sufficient size that the transport medium does not penetrate the central part of the biopsy, which should be used for virus isolation/detection.

1.2. Virus isolation

Lesion material for virus isolation and genome ~~antigen~~ detection is homogenised. The following is an example of one technique for homogenisation: The tissue is minced using sterile scissors and forceps, and then macerated in a steel ball bearing mixer mill or ground with a sterile pestle in a mortar with sterile sand and an equal volume of sterile phosphate buffered saline (PBS) or serum-free Modified Eagle's Medium (MEM) containing sodium penicillin (1000 international units [IU]/ml), streptomycin sulphate (1 mg/ml), mycostatin (100 IU/ml) or fungizone (2.5 µg/ml) and neomycin (200 IU/ml). The homogenised suspension is freeze–thawed three times and then partially clarified by centrifugation using a bench centrifuge at 600 ***g*** for 10 minutes. In cases where bacterial contamination of the sample is expected (such as when virus is isolated from skin samples), the supernatant can be filtered through a 0.45 µm pore size filter after the centrifugation step, however, the amount of virus in the supernatant might be reduced. Buffy coats may be prepared from 5–8 ml unclotted blood by centrifugation at 600 ***g*** for 15 minutes; the buffy coat is carefully removed into 5 ml of cold double-distilled water using a sterile Pasteur pipette. After 30 seconds, 5 ml of cold double-strength growth medium is added and mixed. The mixture is centrifuged at 600 ***g*** for 15 minutes, the supernatant is discarded and the cell pellet is suspended in 5 ml of growth medium, such as Glasgow's modified Eagle's medium (GMEM). After centrifugation at 600 ***g*** for a further 15 minutes, the resulting pellet is suspended in 5 ml of fresh GMEM. Alternatively, the buffy coat may be separated from a heparinised sample using a density gradient.

Capripoxvirus will grow in tissue culture of bovine, ovine or caprine origin, although primary or secondary cultures of lamb testis (LT) or lamb kidney (LK) cells are considered to be the most susceptible. Care needs to be taken to ensure they are not contaminated with viruses such as bovine viral diarrhoea virus~~, particularly those derived from a wool sheep breed~~ (see chapter 1.1.9). Madin–Darby bovine kidney (MDBK) cells have been shown to be suitable for capripoxvirus isolation (Fay *et al.*, 2020). The following is an example of an isolation technique: either 1 ml of buffy coat cell suspension or 1 ml of clarified biopsy preparation supernatant is inoculated on to a 25 cm^2 tissue culture flask of appropriate cells at 90% ~~confluent LT or LK cells~~ confluence, and the supernatant is allowed to adsorb for 1 hour at 37°C. The culture is then washed with warm PBS and covered with 10 ml of a suitable medium, such as GMEM, containing antibiotics and 2% fetal calf serum. If available, tissue culture tubes ~~containing LT or LK cells and a~~, flying cover-slips, or tissue culture microscope slides, ~~are~~ can also infected.

The flasks should be examined daily for 7–14 days for evidence of cytopathic effect (CPE). Contaminated flasks should be discarded. Infected cells develop a characteristic CPE consisting of retraction of the cell membrane from surrounding cells, and eventually rounding of cells and margination of the nuclear chromatin. At first only small areas of CPE can be seen, sometimes as soon as 4 days after infection; over the following 4–6 days these expand to involve the whole cell sheet. If no CPE is apparent by day 7, the culture should be freeze–thawed three times, and clarified supernatant inoculated on to fresh ~~LT or LK~~ cell cultures. At the first sign of CPE in the flasks, or

earlier if a number of infected cover-slips are being used, a cover-slip should be removed, fixed in acetone and stained using H&E. Eosinophilic intracytoplasmic inclusion bodies, which are variable in size but up to half the size of the nucleus and surrounded by a clear halo, are indicative of poxvirus infection. Syncytia formation is not a feature of capripoxvirus infection. If the CPE is due to capripoxvirus infection of the cell culture, it can be prevented or delayed by inclusion of specific anti-capripoxvirus serum in the medium; this provides a presumptive identification of the agent. Some strains of capripoxvirus have been adapted to grow on African green monkey kidney (Vero) cells, but these cells are not recommended for primary isolation.

1.3. Electron microscopy

The characteristic poxvirus virion can be visualised using a negative-staining preparation technique followed by examination with an electron microscope. There are many different negative-staining protocols, an example is given below:

Material from the original tissue suspension is prepared for transmission electron microscope examination, prior to centrifugation, by floating a 400-mesh hexagon electron microscope grid, with piloform-carbon substrate activated by glow discharge in pentylamine vapour, on to a drop of the suspension placed on parafilm or a wax plate. After 1 minute, the grid is transferred to a drop of Tris/EDTA buffer, pH 7.8, for 20 seconds and then to a drop of 1% phosphotungstic acid, pH 7.2, for 10 seconds. The grid is drained using filter paper, air-dried and placed in the electron microscope. The capripoxvirus virion is brick shaped, covered in short tubular elements and measures approximately 290 × 270 nm. A host-cell-derived membrane may surround some of the virions, and as many as possible should be examined to confirm their appearance (Kitching & Smale, 1986).

The virions of capripoxvirus are indistinguishable from those of orthopoxvirus, but, apart from *Vaccinia* virus, no orthopoxvirus causes lesions in sheep and goats. However, capripoxvirus is distinguishable from the virions of parapoxvirus, that cause contagious pustular dermatitis, as they are smaller, oval in shape, and each is covered in a single continuous tubular element, which appears as striations over the virion.

1.4. Histopathology

Material for histopathology and immunohistochemistry should be prepared by standard techniques (Parvin *et al.,* 2022). Following preparation~~,~~ and staining with haematoxylin and eosin (H&E), ~~and mounting of the formalin-fixed biopsy material,~~ a number of sections should be examined by light microscopy. On histological examination, the most striking aspects of acute-stage skin lesions are a massive cellular infiltrate, vasculitis and oedema. Early lesions are characterised by marked perivascular cuffing. Initially infiltration is by macrophages, neutrophils and occasionally eosinophils, and as the lesion progresses, by more macrophages, lymphocytes and plasma cells. A characteristic feature of all capripoxvirus infections is the presence of variable numbers of 'sheep pox cells' in the dermis. These sheep pox cells can also occur in other organs where microscopic lesions of sheep and goat pox are present. These cells are large, stellate cells with eosinophilic, poorly defined intracytoplasmic inclusions and vacuolated nuclei. Vasculitis is accompanied by thrombosis and infarction, causing oedema and necrosis. Epidermal changes consist of acanthosis, parakeratosis and hyperkeratosis. Changes in other organs are similar, with a predominant cellular infiltration and vasculitis. Lesions in the upper respiratory tract are characterised by ulceration.

Immunohistochemistry will show capripox virus antigen infiltrated macrophages throughout the subcutis. The capripox virus antigen can occasionally be detected in hair follicle epithelial cells, the endothelium and smooth muscle cells of the blood vessels, and histiocytic cells (Parvin *et al.,* 2022).

1.5. Immunological methods

1.5.1. Fluorescent antibody tests

Capripoxvirus antigen can also be identified on infected cover-slips or tissue culture slides using fluorescent antibody tests. Cover-slips or slides should be washed and air-dried and fixed in cold acetone for 10 minutes. The indirect test using immune sheep or goat sera is subject to high background colour and nonspecific reactions. However, a direct conjugate can be prepared from sera from convalescent sheep or goats or from rabbits hyperimmunised with purified *Capripoxvirus*. Uninfected tissue culture should be included as a negative control because cross-reactions, due to antibodies to cell culture antigens, can cause problems. The fluorescent antibody tissue section technique has also been used on cryostat-prepared slides.

1.6. Nucleic acid recognition methods

Amplification methods for detection of ~~the viral DNA genome are specific to the genus~~ *Capripoxvirus* DNA are ~~and~~ both specific and sensitive ~~for detection~~ throughout the course of disease, including before and after the emergence of antibody responses. These methods include conventional PCR, real-time PCR, and most recently loop-mediated isothermal amplification (LAMP). Nucleic acid recognition methods can be used to detect the *Capripoxvirus* genome in biopsy, swab, blood, semen or tissue culture samples. It is important that nucleic acid extraction and PCR amplification methods are validated for the sample matrix being tested.

1.6.1. Conventional PCR methods

Several conventional PCR methods have been reported with varying specificity for capripoxviruses in general, SPPV, or GTPV (Heine *et al.,* 1999; Ireland & Binepal, 1998; Zro *et al.,* 2014a). A conventional PCR assay that differentiates GTPV and LSDV from SPPV has been described (Lamien *et al.,* 2011a). Conventional PCR methods are particularly useful for obtaining sufficient genetic material necessary for species identification by subsequent sequence and phylogenetic analysis (Le Goff *et al.,* 2009).

The conventional gel-based PCR method described below is a simple, fast and sensitive method for the detection of capripoxvirus genome in EDTA blood, semen or tissue culture samples (Tuppurainen *et al.,* 2005).

Test procedure

The extraction method described below can be replaced using commercially available DNA extraction kits.

i) Freeze and thaw 200 µl of blood in EDTA, semen or tissue culture supernatant and suspend in 100 µl of lysis buffer containing 5 M guanidine thiocyanate, 50 mM potassium chloride, 10 mM Tris/HCl (pH 8); and 0.5 ml Tween 20.

ii) Cut skin and other tissue samples into fine pieces using a sterile scalpel blade and forceps. Grind with a pestle in a mortar. Suspend the tissue samples in 800 µl of the above mentioned lysis buffer.

iii) Add 2 µl of proteinase K (20 mg/ml) to blood samples and 10 µl of proteinase K (20 mg/ml) to tissue samples. Incubate at 56°C for 2 hours or overnight, followed by heating at 100°C for 10 minutes. Add phenol:chloroform:isoamylalcohol (25:24:1 [v/v]) to the samples in a 1:1 ratio. Vortex and incubate at room temperature for 10 minutes. Centrifuge the samples at 16,060 ***g*** for 15 minutes at 4°C. Carefully collect the upper, aqueous phase (up to 200 µl) and transfer into a clean 2.0 ml tube. Add two volumes of ice cold ethanol (100%) and 1/10 volume of 3 M sodium acetate (pH 5.3). Place the samples at –20°C for 1 hour. Centrifuge again at 16,060 ***g*** for 15 minutes at 4°C and discard the supernatant. Wash the pellets with ice cold 70% ethanol (100 µl) and centrifuge at 16,060 ***g*** for 1 minute at 4°C. Discard the supernatant and dry the pellets thoroughly. Suspend the pellets in 30 µl of nuclease-free water and store immediately at

–20°C (Tuppurainen *et al*., 2005). Alternatively a column-based extraction kit may be used.

iv) The primers for this PCR assay were developed from the gene encoding the viral attachment protein. The size of the expected amplicon is 192 bp (Ireland & Binepal, 1998). The primers have the following gene sequences:

Forward primer 5'-TCC-GAG-CTC-TTT-CCT-GAT-TTT-TCT-TAC-TAT-3'

Reverse primer 5'-TAT-GGT-ACC-TAA-ATT-ATA-TAC-GTA-AAT-AAC-3'.

v) DNA amplification is carried out in a final volume of 50 µl containing: 5 µl of 10 × PCR buffer, 1.5 µl of $MgCl_2$ (50 mM), 1 µl of dNTP (10 mM), 1 µl of forward primer, 1 µl of reverse primer, 1 µl of DNA template (~10 ng), 0.5 µl of *Taq* DNA polymerase and 39 µl of nuclease-free water. The volume of DNA template required may vary and the volume of nuclease-free water must be adjusted to the final volume of 50 µl.

vi) Run the samples in a thermal cycler as follows: 2 minutes at 95°C; then 45 seconds at 95°C, 50 seconds at 50°C and 1 minute at 72°C (34 cycles); 2 minutes at 72°C and hold at 4°C until analysis.

vii) Mix 10 µl of each sample with loading dye and load onto a 1.5% agarose gel in TAE buffer (Tris/acetate buffer containing EDTA). Load a parallel lane with a 100 bp DNA-marker ladder. Electrophoretically separate the products using approximately 8–10 V/cm for 40–60 minutes and visualise with a suitable DNA stain and transilluminator.

1.6.2. Real-time PCR methods

Several highly sensitive and specific fluorescent detection-based real-time PCR methods have been developed and validated (Balinsky *et al*., 2008; Bowden *et al.,* 2008; Das *et al.,* 2012; Stubbs *et al.,* 2012). Each test detects a small conserved genetic locus within the capripoxvirus genome, but these methods do not discriminate between SPPV, GTPV or LSDV. Real-time PCR methods for direct capripoxvirus ~~genotyping~~ species differentiation without the need for gene sequencing have been described (Haegeman *et al.,* 2013; Gelaye *et al.,* 2013; Lamien *et al.,* 2011b; Wolff *et al.,* 2021).

The real-time PCR method described below is a rapid, sensitive and specific method for the detection of the genomic DNA from SPPV, GTPV or LSDV. This assay ~~will~~ is not designed to differentiate ~~between~~ the capripoxvirus species.

DNA extraction from blood, ~~and~~ tissue and semen

A number of DNA extraction kits are commercially available for the ~~isolation~~ extraction of template DNA for real-time PCR. Manufacturer's instructions should always be ~~consulted for guidance on the appropriate method for the sample type being extracted~~ followed while using commercial extraction kits. WOAH Reference Laboratories can be contacted for advice on suitable commercial kits.

Real-time PCR

i) The real-time PCR method outlined below uses the primers and probe described by Bowden *et al*. (2008). and further validated by Stubbs *et al.* (2012). Cycling conditions and reagent concentrations can be altered to ensure optimal performance in individual laboratories.

ii) Forward and reverse primers should be prepared at concentrations of 20 µM. A minor grove binder (MGB) ~~TaqMan~~ hydrolysis probe should be prepared at a concentration of 10 µM.

Forward primer: 5'-AAA-ACG-GTA-TAT-GGA-ATA-GAG-TTG-GAA-3'

Reverse primer: 5'-AAA-TGA-AAC-CAA-TGG-ATG-GGA-TA-3'

Probe: 5'-FAM-TGG-CTC-ATA-GAT-TTC-CT-MGB-3'

iii) Mastermix is prepared by combining 10 µl of 2 × real-time PCR mastermix with 0.4 µl of forward primer, 0.4 µl of reverse primer, 0.5 µl of probe and 6.7 µl of RNase free water per reaction.

iv) Add 2 µl of extracted DNA to 18 µl of mastermix in a 96-well PCR plate or PCR strip and perform real-time PCR according to the example given below or similar method:

v) 95°C for 10 minutes followed by 45 cycles of 95°C for 15 seconds, 60°C for 60 seconds. Fluorescence detection should be performed at the end of each cycle.

vi) Following completion of the real-time PCR, a cycle threshold (C_T) should be set. Samples with C_T values less than 35 are considered positive. Samples with a C_T value greater than 35 but less than 45 are considered inconclusive and require further investigation. Samples which do not yield a C_T value, i.e. the amplification curve does not cross the threshold, are considered negative.

1.6.3. Isothermal genome amplification

Molecular tests using ~~loop-mediated isothermal amplification (~~LAMP~~)~~ to detect capripoxvirus genomes are reported to provide sensitivity and specificity similar to real-time PCR with a simpler method and at lower cost (Das *et al.,* 2012; Murray *et al.,* 2013). Field validation of the Das *et al.* (2012) LAMP ~~method~~ assay has been further reported ~~by~~ (Omoga *et al.,* 2016) and a combination of this universal capripoxvirus test with two additional LAMP assays was reported ~~to show utility in discriminating between~~ to differentiate GTPV ~~and~~ from SPPV (Zhao *et al.,* 2014).

2. Serological tests

Detectable levels of antibodies develop 1 week after the animal shows clinical signs. The highest antibody levels are detected within 1–2 months after infection is detected.

2.1. Virus neutralisation

A test serum can either be titrated against a constant titre of capripoxvirus (100 $TCID_{50}$ [50% tissue culture infective dose]) or a standard capripoxvirus strain can be titrated against a constant dilution of test serum in order to calculate a neutralisation index. Because of the variable sensitivity of tissue culture to capripoxvirus, and the consequent difficulty of ensuring the use of 100 $TCID_{50}$, the neutralisation index is the preferred method, although it does require a larger volume of test sera. The test is described using 96-well flat-bottomed tissue culture grade microtitre plates, but it can be performed equally well in tissue culture tubes with the appropriate changes to the volumes used, although it is more difficult to read an end-point in tubes. ~~The use of Vero cells in the virus neutralisation test has been reported to give more consistent results (Kitching & Taylor, 1985).~~

2.1.1. Test procedure

i) Test sera including a negative and a positive control are diluted 1/5 in Eagle's/HEPES (N-2-hydroxyethylpiperazine, N-2-ethanesulphonic acid) and inactivated at 56°C for 30 minutes.

ii) Next, 50 µl of the first inactivated serum is added to columns 1 and 2, rows A to H of the microtitre plate. The second serum is placed in columns 3 and 4, the third in columns 5 and 6, the positive control serum is placed in columns 7 and 8, the negative control serum is placed in columns 9 and 10, and 50 µl of Eagle's/HEPES without serum is placed in columns 11 and 12 and to all wells of row H.

iii) A reference strain of capripoxvirus, usually a vaccine strain known to grow well in tissue culture, with a titre of over $\log_{10}$ 6 $TCID_{50}$ per ml is diluted in Eagle's/HEPES in bijoux bottles to give a log dilution series of $\log_{10}$ 5.0; 4.0; 3.5; 3.0; 2.5; 2.0; 1.5 $TCID_{50}$ per ml (equivalent to $\log_{10}$ 3.7; 2.7; 2.2; 1.7; 1.2; 0.7; 0.2 $TCID_{50}$ per 50 µl).

iv) Starting with row G and the most diluted virus preparation, 50 µl of virus is added to each well in that row. This is repeated with each virus dilution, the highest titre virus dilution being placed in row A.

v) The plates are covered and incubated for 1 hour at 37°C.

vi) ~~LT cells are~~ An appropriate cell suspension (such as MDBK cells) is prepared from pregrown monolayers as a suspension of 10^5 cells/ml in Eagle's medium containing antibiotics and 2% fetal calf serum. Following incubation of the microtitre plates, 100 µl of cell suspension is added to all the wells, except wells H11 and H12, which serve as control wells for the medium. The remaining wells of row H are cell and serum toxicity controls.

vii) The microtitre plates are covered and incubated at 37°C for 9 days.

viii) Using an inverted microscope, the monolayers are examined daily starting at day 4 for evidence of CPE. There should be no CPE in the cells of row H. Using the 0240 KSGP vaccine strain of capripoxvirus, the final reading is taken on day 9, and the titre of virus in each duplicate titration is calculated according to the Kärber method. If left longer, there is invariably a 'breakthrough' of virus in which virus that was at first neutralised appears to disassociate from the antibody.

ix) *Interpretation of the results:* The neutralisation index is the log titre difference between the titre of the virus in the negative serum and in the test serum. An index of ≥1.5 is positive. The test can be made more sensitive if serum from the same animal is examined before and after infection. Because immunity to capripoxvirus is predominantly cell mediated, a negative result, particularly following vaccination in which the response is necessarily mild, does not imply that the animal from which the serum was taken is not protected.

~~A constant virus/varying serum method has been described using serum dilutions in the range 1/5 to 1/500 and fetal calf muscle cells. Because these cells have a lower sensitivity to capripoxvirus than LT cells, the problem of virus 'breakthrough' is overcome.~~

2.2. Indirect fluorescent antibody test

Capripoxvirus-infected tissue culture grown on flying cover-slips or tissue culture microscope slides can be used for the indirect fluorescent antibody test. Uninfected tissue culture control, and positive and negative control sera, should be included in the test. The infected and control cultures are fixed in acetone at –20°C for 10 minutes and stored at 4°C. Dilutions of test sera are made in PBS, starting at 1/5, and positives are identified using an anti-sheep gamma-globulin conjugated with fluorescein isothiocyanate (Davies & Otema, 1978). Cross-reactions can occur with orf, bovine papular stomatitis virus and perhaps other poxviruses.

2.3. Western blot analysis

Western blotting of test sera against capripoxvirus-infected cell lysate provides a sensitive and specific system for the detection of antibody to capripoxvirus structural proteins, although the test is expensive and difficult to carry out (Chand *et al.*, 1994).

2.4. Enzyme-linked immunosorbent assay

~~No validated ELISA is available for the serological diagnosis of SPP or GTP.~~

Both in-house and commercial enzyme-linked immunosorbent assay (ELISAs) are available, but these tests cannot discriminate between antibodies to different capripoxviruses (LSDV or SPPV/GTPV).

C. REQUIREMENTS FOR VACCINES

[THIS SECTION IS UNDER REVIEW IN THE 2024/2025 REVIEW CYCLE]

1. Background

1.1. Rationale and intended use of the product

A variety of attenuated live and inactivated capripoxvirus vaccines has been used to provide protection against sheeppox and goatpox. All strains of capripoxvirus of ovine, caprine or bovine origin examined so far share a major neutralising site, so that animals recovered from infection with one strain are resistant to infection with any other strain (Capstick, 1961). Consequently, it is possible to use a single strain of capripoxvirus to protect both sheep and goats against all field strains of virus, regardless of whether their origin was in Asia or Africa (Kitching *et al.*, 1986; Kitching & Taylor, 1985). However, field evidence suggests some strains are quite host-specific and are used only in sheep against SPPV and only in goat against GTPV.

A number of strains of capripoxvirus have had widespread use as live vaccines (Davies & Mbugwa, 1985), for example the Romanian and RM-65 strains used mainly in sheep and the Mysore and Gorgan strains used in goats. The real identity of the commonly used Kenyan sheep and goat pox vaccine virus (KSGP) 0240 was recently shown to be actually LSDV (Tuppurainen *et al.,* 2014). Virus strain identity and attenuation properties must be ascertained and taken into consideration when selecting vaccine strains for use in cattle, sheep and goats. The protective dose depends on the vaccine strain used. Immunity in sheep and goats against capripoxvirus following vaccination with the 0240 strain lasts over a year and the Romanian strain gave protection for at least 30 months.

Killed vaccines produced from tissue culture contain only the intracellular mature virion form of the virus, and lack the less robust but biologically crucial extracellular enveloped virion form. As a result, the vaccine does not stimulate immunity against the extracellular enveloped virion, resulting in poor protection. Killed capripoxvirus vaccines provide, at best, only temporary protection.

2. Outline of production and minimum requirements for conventional vaccines

General requirements set for the facilities used for the production of vaccines and for the documentation and record keeping throughout the whole manufacturing process are described in Chapter 1.1.8 *Principles of veterinary vaccine production*. The documentation should include the standard operating procedures (SOP) for the method of manufacture and each step for the testing of cells and reagents used in the process, each batches and the final product.

2.1. Characteristics of the seed

2.1.1. Biological characteristics

A strain of capripoxvirus used for vaccine production must be accompanied by a history describing its origin and tissue culture or animal passage. It must be safe to use in all breeds of sheep and goats for which it is intended, including pregnant and young animals. It must be non-transmissible, remain attenuated after further tissue culture passage, and provide complete protection against challenge with virulent field strains for a minimum of 1 year. A quantity of master seed vaccine virus should be prepared and stored in order to provide a consistent working seed for regular vaccine production.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

Each master seed must be tested to ensure its identity and shown to be free from adventitious viruses, in particular pestiviruses, such as border disease and bovine viral diarrhoea virus, and free from contamination with bacteria, fungi and/or mycoplasmas. The general procedures for sterility or purity tests are described in chapter 1.1.9. The master seed must also be safe and produce no clinical reaction in all breeds of sheep or goats when given by the recommended route and stimulate complete immunity to capripoxvirus in all breeds of sheep and goats for at

least 1 year. The necessary safety and potency tests are described in Section C.2.2.4 *Final product batch tests*.

2.2. Method of manufacture

The method of manufacture should be documented as the Outline of Production.

2.2.1. Procedure

Vaccine seed should be lyophilised and stored in 2 ml vials at –20°C. It may be stored wet at –20°C, but when wet, is more stable at –70°C or lower. The virus should be cultured in primary or secondary LT or LK cells of wool sheep origin for maximum yield. Vero cells may also be used with suitably adapted strains.

Vaccine batches are produced on fresh monolayers of secondary LT or primary LK cells. A vial of seed virus is reconstituted with GMEM or another appropriate medium and inoculated on to an LT or LK monolayer that has been previously washed with warm PBS, and allowed to adsorb for 15 minutes at 37°C before being overlaid with additional GMEM. After 4–6 days, there will be extensive (80–90%) CPE. The culture should be examined for any evidence of nonspecific CPE, medium cloudiness or change in medium pH. The culture is freeze–thawed three times, the suspension removed and centrifuged at 600 ***g*** for 20 minutes. A second passage may be required to produce sufficient virus for a production batch. Live vaccine may be produced on roller bottles.

The procedure is repeated and the harvests from individually numbered flasks are each mixed separately with an equal volume of sterile and chilled 5% lactalbumin hydrolysate and 10% sucrose, and transferred to individually numbered bottles for storage at –20°C. Prior to storage, 0.2 ml is removed from each bottle for sterility control. An additional 0.2 ml is removed for virus titration; 2 ml pools composed of 0.2 ml samples taken from ten bottles are used. A written record of all the procedures must be kept for all vaccine batches.

Inactivated vaccines are produced, usually from unattenuated field strains of capripoxvirus, grown in tissue culture as described above, inactivated with 0.03% formaldehyde, and mixed with an equal volume of alhydrogel as adjuvant. Formaldehyde is no longer considered to be a suitable inactivant for certain viral vaccines because its mode of action cannot be guaranteed to be totally effective in inactivating all the live virus. This has not been fully investigated for capripoxvirus.

2.2.2. Requirements for substrate and media

The specification and source of all ingredients used in the manufacturing procedure should be documented and the freedom from extraneous agents: bacteria, fungi, mycoplasma and any other viruses should be tested. The detailed testing procedure is described in the chapter 1.1.9. The use of antibiotics must meet the requirements of the licensing authority.

2.2.3. In-process controls

i) Cells

Cells should be obtained from the testis or kidney of a healthy young lamb from a scrapie-free flock of a wool sheep breed. During cultivation, cells must be observed for any evidence of CPE, and for normal morphology (predominantly fibroblastic). They can usually be passaged successfully up to ten times. When used for vaccine production, uninfected control cultures should be grown in parallel and maintained for at least three additional passages for further observation. They should be checked for the presence of noncytopathic strains of bovine virus diarrhoea or border disease viruses by immunofluorescence or immunoperoxidase techniques. If possible, cells should be prepared and screened prior to vaccine production and stocked in 1–2 ml aliquots containing 2×10^7 cells/ml in sterile 10% DMSO (dimethyl sulphoxide) and 90% FBS (fetal bovine serum) solution stored in liquid nitrogen.

ii) Serum

Bovine serum used in the growth or maintenance medium must be free from transmissible spongiform encephalopathies (TSEs) and antibody to capripoxvirus, and tested for contamination with pestivirus or any other viruses, extraneous bacteria, mycoplasma or fungi.

iii) Medium

Medium must be tested free from contamination with pestivirus or any other viruses, extraneous bacteria, mycoplasma or fungi.

iv) Virus

Seed virus and final vaccine must be titrated in tissue culture tubes or microtitre plates. Vaccine samples must be examined for the presence of adventitious viruses including cytopathic and noncytopathic strains of pestivirus, and should be mixed with a high-titre capripoxvirus-immune serum that has tested negative for antibody to pestivirus to prevent the vaccine virus itself interfering with the test. The vaccine bulk can be held at –20°C or below until all sterility tests and titrations have been completed, at which time it should be freeze-dried in 1 ml aliquots in vials sufficient for 100 doses. The vaccine harvest diluted with lactalbumin hydrolysate and sucrose should have a minimum titre $\log_{10}$ 4.5 $TCID_{50}$ per ml after freeze-drying, equivalent to a field dose of $\log_{10}$ 2.5 $TCID_{50}$. A further titration is carried out on five randomly chosen vials of the freeze-dried preparation to confirm the titre.

2.2.4. Final product batch tests

i) Sterility/purity

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

ii) Safety

The safety studies should be demonstrated by statistically valid vaccination studies using seronegative young sheep and goats of known susceptibility to capripox virus. The procedure described is suitable for vaccine strains such as 0240 that are equally immunogenic in both sheep and goats. The choice of target animal should be adapted for strains with a more restricted host preference.

iii) Potency

Potency tests must be undertaken if the minimum immunising dose of the virus strain is not known. This is usually carried out by comparing the titre of a virulent challenge virus on the flanks of vaccinated and control animals. Following vaccination, the flanks of at least three animals and three controls are shaved of wool or hair. Log_{10} dilutions of the challenge virus are prepared in sterile PBS and six dilutions are inoculated intradermally (0.1 ml per inoculum) along the length of the flank; four replicates of each dilution are inoculated down the flank. An oedematous swelling will develop at possibly all 24 inoculation sites on the control animals, although preferably there will be little or no reaction at the four sites of the most dilute inocula. The vaccinated animals should develop an initial hypersensitivity reaction at sites of inoculation within 24 hours, which should quickly subside. Small areas of necrosis may develop at the inoculation site of the most concentrated challenge virus. The macule/papule is measured at between 8 and 10 days post-challenge. The titre of the challenge virus is calculated for the vaccinated and control animals; a difference of $\log_{10}$ titre > 2.5 is taken as evidence of protection.

2.3. Requirements for authorisation

2.3.1. Safety requirements

i) Target and non-target animal safety

The vaccine must be safe to use in all breeds of sheep and goats for which it is intended, including young and pregnant animals. It must also be non-transmissible, remain attenuated after further tissue culture passage.

Safety tests should be carried out on the final product of each batch as described in Section C.2.2.4.

The safety of the vaccine in non-target animals must have been demonstrated using mice and guinea-pigs as described in Section C.2.2.4. There should be no evidence of pathology caused by the vaccine.

ii) Reversion-to-virulence for attenuated/live vaccines

The selected final vaccine should not revert to virulence during a further passages in target animals.

iii) Environmental consideration

Attenuated vaccine should not be able to perpetuate autonomously in cattle, sheep or goat populations. Vaccines using the 0240 strain should not be used in *Bos taurus* breeds. Strains of capripoxvirus are not a hazard to human health. There are no precautions other than those described above for sterility and freedom from adventitious agents.

2.3.2. Efficacy requirements

i) For animal production

The efficacy of the vaccine must be demonstrated in vaccination challenge experiment under laboratory conditions. As described in Section C.2.2.4.

Once the potency of the particular strain being used for vaccine production has been determined in terms of minimum dose required to provide immunity, it is not necessary to repeat this on the final product of each batch, provided the titre of virus present has been ascertained.

ii) For control and eradication

Vaccination is the only effective way to control the sheep pox and goat pox outbreaks in endemic countries. Unfortunately, currently no marker vaccines allowing the differentiation of infected from vaccinated animals are available.

Immunity to virulent field virus following vaccination of sheep or goats with the 0240 strain lasts over 1 year, and protection against generalised infection following intradermal challenge lasts at least 3 years and is effective lifelong. The duration of immunity produced by other vaccine strains should be ascertained in both sheep and goats by undertaking controlled trials in an environment in which there is no possibility of field strains of capripoxvirus confusing the results. The inactivated vaccines provide immunity for less than 1 year, and for the reasons given at the beginning of this section, may not give immunity to the form of capripoxvirus usually associated with natural transmission.

2.3.3. Stability

All vaccines are initially given a shelf-life of 24 months before expiry. Real-time stability studies are then conducted to confirm the appropriateness of the expiry date. Multiple batches of the vaccine should be re-titrated periodically throughout the shelf-life to determine the vaccine variability.

Properly freeze-dried preparations of capripox vaccine, particularly those that include a protectant, such as sucrose and lactalbumin hydrolysate, are stable for over 25 years when stored at –20°C and for 2–4 years when stored at 4°C. There is evidence that they are stable at higher temperatures, but no long-term controlled experiments have been reported. The inactivated vaccines must be stored at 4°C, and their shelf- life is usually given as 1 year.

No preservatives other than a protectant, such as sucrose and lactalbumin hydrolysate, are required for the freeze-dried preparation.

3. Vaccines based on biotechnology

3.1. Vaccines available and their advantages

Currently, no recombinant vaccines for capripoxviruses are commercially available. However, a new generation of capripox vaccines is being developed that uses the capripoxvirus genome as a vector for the genes of other ruminant pathogens such as peste des petits ruminants (PPR) virus (Berhe *et al.*, 2003; Tuppurainen *et al*., 2014).

3.2. Special requirements for biotechological vaccines, if any

Not applicable.

REFERENCES

BALINSKY C.A, DELHON G, SMOLIGA G, PRARAT M, FRENCH R.A, GEARY S.J, ROCK D.L & RODRIGUEZ L.L. (2008). Rapid preclinical detection of sheep pox virus by a real-time PCR assay. *J. Clin. Microbiol.,* **46**, 438–442.

BERHE G., MINET C., LE GOFF C., BARRETT T., NGANGNOU A., GRILLET C., LIBEAU G., FLEMING M., BLACK D.N. & DIALLO A. **(2003).** Development of a dual recombinant vaccine to protect small ruminants against peste-des-petits-ruminants virus and capripoxvirus infections. *J. Virol*., **77**, 1571–1577.

BOWDEN T.R, BABIUK S.L, PARKYN G.R., COPPS J.S. & BOYLE D.B. (2008). Capripox virus tissue tropism and shedding: A quantitative study in experimentally infected sheep and goats. *Virology* , **371**, 380–393.

CAPSTICK P.B. (1961). Annual Report. Kenya Veterinary Department, Kenya, 45–47.

CHAND P., KITCHING R.P. & BLACK D.N. (1994). Western blot analysis of virus-specific antibody responses to capripoxvirus and contagious pustular dermatitis infections in sheep. *Epidemiol. Infect*., **113**, 377–385.

DAS A., BABIUK S. & MCINTOSH M.T. (2012). Development of a loop-mediated isothermal amplification assay for rapid detection of capripoxviruses. *J. Clin. Microbiol*., **50**, 1613–1620.

DAVIES F.G. & MBUGWA G. (1985). The alterations in pathogenicity and immunogenicity of a Kenya sheep and goat pox virus on serial passage in bovine foetal muscle cell cultures. *J. Comp. Pathol*., **95**, 565–576.

DAVIES F.G. & OTEMA C. (1978). The antibody response in sheep infected with a Kenyan sheep and goat pox virus. *J. Comp. Pathol*., **88**, 205–210.

Fay P.C., Cook C.G., Wijesiriwardana N., Tore G., Comtet L., Carpentier A., Shih B., Freimanis G., Haga I.R. & Beard P.M. (2020). Madin–Darby bovine kidney (MDBK) cells are a suitable cell line for the propagation and study of the bovine poxvirus lumpy skin disease virus. *J. Virol. Methods*, **285**, 113943. doi: 10.1016/j.jviromet.2020.113943.

GELAYE E., LAMIEN C.E., SILBER R., TUPPURAINEN E.S., GRABHERR R. & DIALLO A.(2013). Development of a cost-effective method for capripoxvirus genotyping using snapback primer and dsDNA intercalating dye. *PLoS One*, **8** (10): e75971.

HAEGEMAN A., ZRO K., VANDENBUSSCHE F., DEMEESTERE L., VAN CAMPE W., ENNAJI M.M. & DE CLERCQ K. (2013). Development and validation of three Capripoxvirus real-time PCRs for parallel testing. *J. Virol.Methods*, **193**, 446–451. doi: 10.1016/j.jviromet.2013.07.010. Epub 2013 Jul 11.

HEINE H.G., STEVENS M.P., FOORD A.J. & BOYLE D.B. (1999). A capripoxvirus detection PCR and antibody ELISA based on the major antigen P32, the homolog of the vaccinia virus H3L gene. *J. Immunol. Methods*, **227**, 187–196.

IRELAND D.C. & BINEPAL Y.S. (1998). Improved detection of capripoxvirus in biopsy samples by PCR. *J. Virol. Methods*, **74**, 1–7.

KITCHING R.P., HAMMOND J.M. & TAYLOR W.P. (1986). A single vaccine for the control of capripox infection in sheep and goats. *Res. Vet. Sci.,* **42**, 53–60.

KITCHING R.P. & SMALE C. (1986). Comparison of the external dimensions of capripoxvirus isolates. *Res. Vet. Sci.*, **41**, 425–427.

KITCHING R.P. & TAYLOR W.P. (1985). Clinical and antigenic relationship between isolates of sheep and goat pox viruses. *Trop. Anim. Health Prod.*, **17**, 64–74.

LAMIEN C.E., GOFF C.L., SILBER R., WALLACE D.B., GULYAZ V., TUPPURAINEN E., MADANI H., CAUFOUR P., ADAM T., EL HARRAK M., LUCKINS A.G., ALBINA E. & DIALLO A. (2011a). Use of the Capripoxvirus homologue of Vaccinia virus 30kDa RNA polymerase subunit (RPO30) gene as a novel diagnostic and genotyping target: development of a classical PCR method to differentiate Goat poxvirus from Sheep poxvirus. *Vet. Microbiol.*, **149**, 30–39. doi: 10.1016/j.vetmic.2010.09.038.

LAMIEN C.E., LELENTA M., GOGER W., SILBER R., TUPPURAINEN E., MATIJEVIC M., LUCKINS A.G. & DIALLO A. (2011b). Real time PCR method for simultaneous detection, quantitation and differentiation of capripoxviruses. *J. Virol. Methods*, **171**, 134–140.

LE GOFF C., LAMIEN C.E., FAKHFAFH E., CHADEYRAS A., ABU-ADULUGBAD E., LIBEAU G., TUPPURAINEN E., WALLACE D., ADAM T., SILBER R., GULYAZ V., MADANI H., CAUFOUR P., HAMAMMI S., DIALLO A. & ALBINA E. (2009). Capripoxvirus G-protein-coupled chemokine receptor, a host-range gene suitable for virus-animal origin discrimination. *J. Gen. Virol.*, **90**, 67–77.

MURRAY L., EDWARDS L., TUPPURAINEN E.S., BACHANEK-BANKOWSKA K., OURA C.A., MIOULET V. & KING D.P. (2013). Detection of capripoxvirus DNA using a novel loop-mediated isothermal amplification assay. *BMC Vet. Res.*, **9**, 90.

OMOGA D.C.A., MACHARIA M., MAGIRI E., KINYUA J., KASIITI J. & HOLTON T. (2016) Molecular based detection, validation of a LAMP assay and phylogenetic analysis of capripoxvirus in Kenya. *J. Adv. Biol. Biotech.,* **7**, 1–12.

PARVIN R., CHOWDHURY E.H., ISLAM M.T., BEGUM J.A., NOORUZZAMAN M., GLOBIG A., DIETZE K., HOFFMANN B. & TUPPURAINEN E. (2022). Clinical Epidemiology, Pathology, and Molecular Investigation of Lumpy Skin Disease Outbreaks in Bangladesh during 2020–2021 Indicate the Re-Emergence of an Old African Strain. *Viruses*, **14**, 2529. doi: 10.3390/v14112529. PMID: 36423138; PMCID: PMC9698944.

STUBBS S., OURA C.A., HENSTOCK M., BOWDEN T.R., KING D.P. & TUPPURAINEN E.S. (2012). Validation of a high-throughput real-time polymerase chain reaction assay for the detection of capripoxviral DNA. *J. Virol. Methods*, **179**, 419–422.

TUPPURAINEN E.S.M., PEARSON C.R., BACHANEK-BANKOWSKA K., KNOWLES N.J., AMAREEN S., FROST L., HENSTOCK M.R., LAMIEN C.E., DIALLO A. & MERTENS P.P.C. (2014). Characterization of sheep pox virus vaccine for cattle against lumpy skin disease virus. *Antiviral Res.*, **109**, 1–6.

TUPPURAINEN E.S.M., VENTER E.H. & COETZER J.A.W. (2005). The detection of lumpy skin disease virus in samples of experimentally infected cattle using different diagnostic techniques. *Onderstepoort J. Vet. Res.*, **72**, 153–164.

WOLFF J.,& BEER M. & Hoffmann B. (2021). Probe-Based Real-Time qPCR Assays for a Reliable Differentiation of Capripox Virus Species. *Microorganisms*, **9**, 765. https://doi.org/10.3390/microorganisms9040765

ZHAO Z., FAN B., WU G., YAN X., LI Y., ZHOU X., YUE H., DAI X., ZHU H., TIAN B., LI J. & ZHANG Q. (2014) Development of loop-mediated isothermal amplification assay for specific and rapid detection of differential goat Pox virus and Sheep Pox virus. *BMC Microbiol.,* **14**, 1–10.

ZRO K., AZELMAT S., BENDOURO Y., KUHN J.H., EL FAHIME E. & ENNAJI M.M. (2014a). PCR-based assay to detect sheeppox virus in ocular, nasal, and rectal swabs from infected Moroccan sheep. *J. Virol. Methods*, **204**, 38–43.

ZRO K., ZAKHAM F., MELLOUL M., EL FAHIME E. & MUSTAPHA M. (2014b). A sheeppox outbreak in Morocco: isolation and identification of virus responsible for the new clinical form of disease. *BMC Vet Res*., **10**, 31.

*

* *

NB: There are WOAH Reference Laboratories for sheep pox and goat pox (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 sheep pox and goat pox

NB: FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2017.

Annex 16. Chapter 3.9.1. 'African swine fever (infection with African swine fever virus)'

SECTION 3.9.

SUIDAE

CHAPTER 3.9.1.

AFRICAN SWINE FEVER (INFECTION WITH AFRICAN SWINE FEVER VIRUS)

SUMMARY

African swine fever (*ASF*) *is an infectious disease of domestic and wild pigs of all breeds and ages, caused by ASF virus* (*ASFV*). *The clinical syndromes vary from peracute, acute, subacute to chronic, depending on the virulence of the virus. Acute disease is characterised by high fever, haemorrhages in the reticuloendothelial system, and a high mortality rate. Soft ticks of the* Ornithodoros *genus, especially* O. moubata *and* O. erraticus, *have been shown to be both reservoirs and transmission vectors of ASFV. The virus is present in tick salivary glands and passed to new hosts* (*domestic or wild suids*) *when feeding. It can be transmitted sexually between ticks, transovarially to the eggs, or transtadially throughout the tick's life.*

ASFV is the only member of the Asfarviridae *family, genus* Asfivirus.

Laboratory diagnostic procedures for ASF fall into two groups: detection of the virus and serology. The selection of the tests to be carried out depends on the disease situation and laboratory diagnostic capacity in the area or country.

Identification of the agent: *Laboratory diagnosis must be directed towards isolation of the virus by inoculation of pig leukocyte or bone marrow cultures, the detection of antigen in smears or cryostat sections of tissues by fluorescent antibody test and/or the detection of genomic DNA by the polymerase chain reaction* (*PCR*) *or real-time PCR. The PCRs are excellent, highly sensitive, specific and rapid techniques for ASFV detection and are very useful under a wide range of circumstances. They are especially useful if the tissues are unsuitable for virus isolation and antigen detection. In doubtful cases, the material is passaged in leukocyte cell cultures and the procedures described above are repeated.*

Serological tests: *Pigs that survive natural infection usually develop antibodies against ASFV from 7–10 days post-infection and these antibodies persist for long periods of time. Where the disease is endemic, or where a primary outbreak is caused by a strain of low or moderate virulence, the investigation of new outbreaks should include the detection of specific antibodies in serum or extracts of the tissues submitted. A variety of methods such as the enzyme-linked immunosorbent assay* (*ELISA*), *the indirect fluorescent antibody test* (*IFAT*), *the indirect immunoperoxidase test* (*IPT*), *and the immunoblotting test* (*IBT*) *is available for antibody detection.*

Requirements for vaccines: ~~*At present, there is no vaccine for ASF.*~~ *Commercially produced modified live virus vaccines are* ~~*available and licenced*~~ *under field evaluation in some countries.*

A. INTRODUCTION

The current distribution of African swine fever (ASF) extends across more than 50 countries in three continents (Africa, Asia and Europe). Several incursions of ASF out of Africa were reported between the 1960s and 1970s. In 2007, ASF was introduced into Georgia, from where it spread to neighbouring countries including the Russian Federation. From there ASF spread to eastern European countries extending westwards and reaching the European Union in 2014. Further westward and southern spread in Europe has occurred since that time. In all these countries, both hosts – domestic pig and wild boar – were affected by the disease. In August 2018, the People's Republic of China reported its first outbreak of ASF and further spread in Asia has occurred. ASF was identified on the island of Hispaniola (Haiti and the Dominican Republic) in 2021. See WAHIS (https://wahis.woah.org/#/home) for recent information on distribution at the country level.

ASF virus (ASFV) is a complex large, enveloped DNA virus with icosahedral morphology. It is currently classified as the only member of the *Asfaviridae* family, genus *Asfivirus* (Dixon *et al.*, 2005). More than 60 structural proteins have been identified in intracellular virus particles (200 nm) (Alejo *et al.,* 2018). More than a hundred infection-associated proteins have been identified in infected porcine macrophages, and at least 50 of them react with sera from infected or recovered pigs (Sánchez-Vizcaíno & Arias, 2012). The ASFV double-stranded linear DNA genome comprises between 170 and 193 kilobases (kb) and contains between 150 and 167 open reading frames with a conserved central region of about 125 kb and variable ends. These variable regions encode five multigene families that contribute to the variability of the virus genome. The complete genomes of several ASFV strains have been sequenced (Bishop *et al.,* 2015; Chapman *et al.,* 2011; de Villiers *et al.,* 2010; Portugal *et al.,* 2015). Different strains of ASFV vary in their ability to cause disease, but at present there is only one recognised serotype of the virus detectable by antibody tests.

The molecular epidemiology of the disease is investigated by sequencing of the 3' terminal end of the B646L open reading frame encoding the p72 protein major capsid protein, which differentiates up to 24 distinct genotypes (Achenbach *et al*., 2017; Boshoff *et al.,* 2007; Quembo *et al.* 2018). To distinguish subgroups among closely related ASFV, sequence analysis of the tandem repeat sequences (TRS), located in the central variable region (CVR) within the B602L gene (Gallardo *et al.,* 2009; Lubisi *et al.,* 2005; Nix *et al.,* 2006) and in the intergenic region between the I73R and I329L genes, at the right end of the genome (Gallardo *et al.,* 2014), is undertaken. Several other gene regions such as the E183L encoding p54 protein, the CP204L encoding p30 protein, and the protein encoded by the EP402R gene (CD2v), have been proved as useful tools to analyse ASFVs from different locations and hence track virus spread.

ASF viruses produce a range of syndromes varying from peracute, acute to chronic disease and subclinical infections. Pigs are the only domestic animal species that is naturally infected by ASFV. European wild boar and feral pigs are also susceptible to the disease, exhibiting clinical signs and mortality rates similar to those observed in domestic pigs. In contrast African wild pigs such as warthogs (*Phacochoerus aethiopicus*), bush pigs (*Potamochoerus porcus*) and giant forest hogs (*Hylochoerus meinertzhageni*) are resistant to the disease and show few or no clinical signs. These species of wild pig act as reservoir hosts of ASFV in Africa (Costard *et al.,* 2013; Sánchez-Vizcaíno *et al.,* 2015).

The incubation period is usually 4–19 days. The more virulent strains produce peracute or acute haemorrhagic disease characterised by high fever, loss of appetite, haemorrhages in the skin and internal organs, and death in 4–10 days, sometimes even before the first clinical signs are observed. Case fatality rates may be as high as 100%. Less virulent strains produce mild clinical signs – slight fever, reduced appetite and depression – which can be readily confused with many other conditions in pigs and may not lead to suspicion of ASF. Moderately virulent strains are recognised that induce variable disease forms, ranging from acute to subacute. Low virulence, non-haemadsorbing strains can produce subclinical non-haemorrhagic infection and seroconversion, but some animals may develop discrete lesions in the lungs or on the skin in areas over bony protrusions and other areas subject to trauma. Animals that have recovered from either acute, subacute or chronic infections may potentially

become persistently infected, acting as virus carriers. The biological basis for the persistence of ASFV is still not well understood, nor it is clear what role persistence plays in the epidemiology of the disease.

ASF cannot be differentiated from classical swine fever (CSF) by either clinical or post-mortem examination, and both diseases should be considered in the differential diagnosis of any acute febrile haemorrhagic syndrome of pigs. Bacterial septicaemias may also be confused with ASF and CSF. Laboratory tests are essential to distinguish between these diseases.

In countries free from ASF but suspecting its presence, the laboratory diagnosis must be directed towards isolation of the virus by the inoculation of pig leukocyte or bone marrow cultures, the detection of genomic DNA by polymerase chain reaction (PCR) or the detection of antigen in smears or cryostat sections of tissues by direct fluorescent antibody test (FAT). Currently the PCR is the most sensitive technique and can detect ASFV DNA from a very early stage of infection in tissues, ethylene diamine tetra-acetic acid (EDTA)-blood and serum samples. The PCR is particularly useful if samples submitted are unsuitable for virus isolation and antigen detection because they have undergone putrefaction. Pigs that have recovered from acute, subacute or chronic infections usually exhibit a viraemia for several weeks making the PCR test a very useful tool for the detection of ASFV DNA in pigs infected with low or moderately virulent strains. Virus isolation by the inoculation of pig leukocyte or bone marrow cultures and identification by haemadsorption tests (HAD) are recommended as a confirmatory test when ASF is positive by other methods, particularly in the event of a primary outbreak or a case of ASF.

~~As no vaccine is available, the presence of ASFV antibodies is indicative of previous infection and, as antibodies are produced from the first week of infection and persist for long periods, they are a good marker for the diagnosis of the disease, particularly in subacute and chronic forms.~~

Vaccines should be prepared in accordance with Chapter 1.1.8 *Principles of veterinary vaccine production*. Current ASF modified live virus (MLVs) vaccines are based on the live virus that have been naturally attenuated or attenuated by targeted genetic recombination through cell cultures (Gladue & Borca, 2022). MLV production is based on a seed-lot system consistent with the *European Pharmacopoeia* (11th edition) and that has been validated with respect to virus identity, sterility, purity, potency, stability, safety and immunogenicity (including spread)~~, non-transmissibility, stability and immunogenicity~~. ASF MLV first generation vaccines ~~– defined as those for which peer-reviewed publications are in the public domain –~~ should meet or exceed the minimum standards as described below. ~~Paramount~~ Demonstration of acceptable safety and efficacy against the epidemiologically relevant ASFV field strain(s) circulating in areas where the vaccine is intended for use ~~are~~ is required. At the present time, a variety of mutants (Forth *et al.,* 2023) and recombinants (Zhao *et al.,* 2023) have emerged globally, and the prevalence of these strains is increasing. In addition, there is a risk that vaccine strains will recombine with circulating strains. These conditions should be taken into account in vaccine development. ~~acceptable efficacy should be shown against the B646L (p72) genotype II pandemic virus lineage currently circulating widely in domestic pigs and wild boar.~~

ASF MLV first generation vaccines allowing the differentiation of infected animals from vaccinated animals (DIVA) by suitable methods (e.g. serology-based tests) are preferred. Demonstration of MLV safety and efficacy in pigs at different growth stages (suckling piglets, nursery pigs, fattening pigs), the safety in breeding-age boars, gilts and pregnant sows, ~~and onset and duration of protective immunity,~~ are also preferred~~,~~ but are not required to meet the minimum standard. Additional data will likely be required by Regulatory Authorities if these categories are included in the indications for the vaccine. Details of the onset of immunity (the interval of time elapsed between vaccination and challenge if protection is confirmed) and the duration of immunity (the time point at which vaccine-induced immunity begins to decline and provides less protection) are also required to meet minimum standards.

ASF epidemiology is complex with different epidemiological patterns of infection occurring in Africa and Europe. ASF occurs through transmission cycles involving domestic pigs, wild boar, wild African suids, and soft ticks (Sánchez-Vizcaíno *et al.,* 2015). In regions where *Ornithodoros* ~~soft-bodied~~ ticks are present, the detection of ASFV in these reservoirs of infection contributes to a better understanding of the epidemiology of the disease. This is of major importance in establishing effective control and eradication programmes (Costard *et al*., 2013).

ASF is not a zoonotic disease and does not affect public health (Sánchez-Vizcaíno *et al.*, 2009).

ASFV should be handled with an appropriate level of bio-containment, determined by risk analysis in accordance with Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*.

C. REQUIREMENTS FOR VACCINES [UNDER REVIEW]

~~At present there is no commercially available vaccine for ASF.~~ Commercially produced modified live virus vaccines are being evaluated and licensed for field use.

1. Background

The ASF p72 genotype II strains (ASFV Georgia 2007/1 lineage) (NCBI, 2020) are recognised to be the current highest global threat for domestic pig production worldwide (Penrith *et al.*, 2022). However, genotype I attenuated strains and genotype I/II recombinant strains have been reported to be circulating. In Africa, multiple genotypes are circulating.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of Veterinary Vaccine Production*. Varying additional requirements relating to quality (including purity and potency), safety, and efficacy will apply in particular countries or regions for manufacturers to comply with local regulatory requirements.

Wherever live, virulent ASFV or ASF MLVs are stored, handled and disposed, the appropriate biosecurity level, procedures and practices should be used. The ASF MLV vaccine production facility should meet the requirements for containment outlined in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*.

An optimal ASF MLV first generation vaccine for the target host should have the following general characteristics (minimum standards):

- Safe: demonstrate absence of persistent fever as defined below (see Section 2.3.2) and clinical signs of acute or chronic ASF in vaccinated and in-contact animals, minimal and ideally no vaccine virus transmission, and absence of an increase in virulence (genetic and phenotypic stability);
- Efficacious: protects against mortality, reduces acute disease (fever accompanied by the appearance of clinical signs caused by ASF) and ~~reduces vertical (boar semen and placental) and~~ horizontal disease transmission;
- Quality – purity: free from wild-type ASFV and extraneous microorganisms that could adversely affect the safety, potency or efficacy of the product;
- Quality – ~~potent~~ stability: the ~~log$_{10}$~~ virus titre maintained throughout the vaccine shelf life that guarantees the efficacy demonstrated by the established minimum immunising (protective) dose.
- ~~Identity~~ Vaccine matching: based on the capacity to protect against the ASFV B646L (p72) genotype II pandemic strain or other p72 genotypes of recognised epidemiologic importance.

Vaccine production should be carried out using a validated, controlled and consistent manufacturing process.

ASF MLV first generation vaccines must be safe (i.e. an acceptable safety profile) for non-target species and the environment in general.

Ideally, ASF MLV first generation vaccines that meet the minimum standards should also fulfil the following additional general characteristics: i) prevents acute and persistent ~~(carrier state)~~ disease; ii) prevents horizontal and vertical disease transmission; iii) induces rapid protective immunity (e.g. < 2 weeks); and iv) confers stable, life-long immunity.

Furthermore, ASF MLV second and future generation vaccines should meet the minimum safety and efficacy standards as ASF MLV first generation vaccines, and ideally provide additional product profile benefits, including but not limited to: i) contain a negative marker allowing the differentiation of infected

from vaccinated animals (DIVA) by reliable discriminatory tests such as serology-based tests; and ii) confer broad range of protection against other p72 genotype field strains of varying virulence (low, moderate, and high).

The majority of ASF global vaccine research groups and companies are currently focused on ASF MLV first generation vaccine candidates that are safe and efficacious against ASF viruses belonging to the ASFV p72 genotype II pandemic strain (ASFV Georgia 2007/1 lineage) (NCBI, 2020). More research is needed to determine whether these genotype II-specific MLVs can effectively protect against newly circulating variants of genotype II and recombinant strains.

Currently, two recombinant gene deleted MLV ~~recombinant~~ vaccines (ASFV-G-ΔI177L and ASFV-G-ΔMGF) have been licenced ~~for field use~~ in Vietnam for use in domestic pigs ~~following supervised field testing to evaluate the safety and effectiveness of several vaccine batches~~.

There are numerous, promising ASF MLV vaccine candidates targeting the p72 genotype II pandemic strain under development, including:

- A naturally attenuated field strain (Lv17/WB/Rei1) (Barasona *et al.,* 2019) being developed as an oral bait vaccine for wild boar~~s~~;
- A laboratory thermo-attenuated field strain (ASFV-989) (Bourry *et al.,* 2022);
- Single gene-deleted, recombinant viruses (e.g. SY18ΔI226R, ASFV-G-ΔA137R) (Gladue *et al*., 2021; Zhang *et al*., 2021);
- Double gene-deleted, recombinant viruses (e.g. ASFV-G-Δ9GL/ΔUK; ASFV-SY18-ΔCD2v/UK; Arm-ΔCD2v-ΔA238L) (O'Donnell *et al*., 2016; Pérez-Núñez *et al*., 2022; Teklue *et al*., 2020);
- Multiple gene-deleted, recombinant viruses (ASFV-G-ΔI177L/ΔLVR; ASFV-G-ΔMGF; ~~BA71ΔCD2;~~ HLJ/18-7GD; ASFVGZΔI177LΔCD2vΔMGF) (Borca *et al*., 2021; Chen *et al*., 2020; Kitamura *et al*., 2023; Liu *et al*., 2023; Monteagudo *et al*., 2017; O'Donnell *et al*., 2015).

Information regarding many of these MLV vaccine candidates can be found in a recent review publication (Brake, 2022).

Different DIVA strategies using serological methods (e.g. ELISA) or genome detection methods (e.g. differential real-time PCR) are not widely available for these ASF MLV first generation vaccine candidates. Therefore, there is still room for improvement with respect to marker vaccines and their companion diagnostic tests.

Inactivated (non-replicating) whole virus vaccines are not presently available and may be difficult to develop to meet minimum efficacy standards. Recombinant vectored, subunit vaccine candidates that can be produced in scalable vaccine platform expression systems and mRNA-based ASF vaccines are being evaluated in ongoing laboratory research, testing and evaluation in experimental challenge models. The publicly available *Center of Excellence for African Swine Fever Genomics* (ASFV Genomics, 2022[1]) that provides the structural protein predictions for all 193 ASFV proteins may help accelerate ASF first and second generation vaccine research and development.

Any future use of vaccine candidates should be based on a thorough risk–benefit assessment considering all safety and efficacy features, as well as the potential vaccination scenario. Fit-for-purpose vaccine use scenarios matched to the intended use in a domestic pig-specific type of production system may require different vaccine product profiles or may influence the focus of essential versus ideal vaccine requirements. Prudent use of ASF MLVs as part of strict, controlled vaccination programmes, especially in the areas where ASF is not prevalent, should be implemented.

It is important to know what genotypes of ASFV are circulating in a population before vaccination is introduced. Due to the potential risk of recombination events between circulating low and high virulent field strains with future licensed vaccine strains, and the possibility of reversion to virulence of vaccine strains, strict pharmacovigilance post-vaccination is essential. Field pharmacovigilance data should be collected and analysed during vaccination campaigns using ASF MLV first generation vaccines post-

[1] http://asfvgenomics.com. Accessed 4/4/2023.

licensing. Active post-vaccination surveillance programmes for the detection of new ASF viruses that may arise from MLV vaccine strains and naturally circulating wild-type virus recombination, as well as revertant vaccine strains, should be implemented. It is also recommended that vaccine manufacturers carry out laboratory experiments to further evaluate the risk of vaccine virus recombination with field and vaccine strains.

As with any ~~MLV~~ vaccine, all ASF ~~MLV~~ vaccines should be used according to the label instructions, under the strict control of the country's Regulatory Authority.

The minimum standards given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national, regional, and veterinary international medicinal product harmonised requirements. Minimum data requirements for an authorisation in exceptional circumstances (e.g. unexpected introduction of the virus, sudden outbreaks of the disease) should be considered where applicable.

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed virus

2.1.1. Biological characteristics of the master seed virus

ASF MLVs are generally produced from ASFV field strains derived from naturally attenuated field isolates or using DNA homologous (genetically targeted) recombination techniques in cell cultures to delete one or more ASFV genes or gene families. These molecular techniques typically involve replacement of the targeted ASFV gene(s) with one or more positive, marker fluorescent (e.g. BFP, eGFP, mCherry) or enzyme-based (e.g. β-glucuronidase) ASFV promoter-reporter gene systems that allow the use of imaging microscopy or flow cytometry to visualise, select, and clone gene-deleted, recombinant, ASF MLVs. MLV production is carried out in cell cultures based on a seed-lot system.

Master seed viruses (MSVs) for MLVs should be selected and produced based on their ease of growth in cell culture, virus yield (log_{10} infectious titre) and genetic stability over multiple cell passages. Preferably, a continuous well-characterised cell line (e.g. ZMAC-4; PIPEC; IPKM) (Borca *et al.*, 2021; Masujin *et al.*, 2021; Portugal *et al.*, 2020) is used to produce a master cell bank (MCB) on which the MSV and MSV-derived working seed virus (WSV) can be produced. The exact source of the underlying ASFV isolate, the whole genome sequence, and the passage history must be recorded.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

Only MSVs that have been established as sterile, pure (free of wild-type parental virus and free of extraneous agents as described in Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*, and those listed by the appropriate licensing authorities) and immunogenic, should be used as the vaccine virus (WSV and vaccine batch production). ~~Live vaccines must be shown not to cause disease or other adverse effects in target animals in accordance with chapter 1.1.8, Section 7.1 *Safety tests* (for live attenuated MSVs), that includes target animal safety tests, increase in virulence tests, assessing the risk to the environment) and if possible, no transmission to other animals.~~

Identity of the MSV must be confirmed using appropriate methods (e.g. through the use of vaccine strain-specific whole genome detection methods such as next generation sequencing).

Demonstration of MSV stability over several cell passages is necessary, typically through at least five passages (e.g. MSV+5). For those MLV vaccines for which attenuation is linked to specific characteristics (gene deletion, gene mutations, etc.), genetic stability of attenuation throughout the production process should be confirmed by full genome sequencing and confirmation of the vaccine phenotype, for example, by confirming the virus titre obtained by growth in the cell line used for production ~~using~~

~~suitable methods. Suitable techniques to demonstrate genetic stability may include but are not limited to: genome sequencing, biochemical, proteomic, genotypic (e.g. detection of genetic markers) and phenotypic strain characterisation~~. If final product yields (infectious titres) are relatively low, as is typically the case with ASFV, demonstration of stability is required for the maximum passage for use in the final product manufacturing as defined by the producer ~~genetic stability at a minimum of MSV+10 should be demonstrated to allow more flexibility in the outline of production. For example, if MSV+8 is the maximum passage for use in final product manufacturing, demonstration of genetic stability to at least MSV+10 is warranted~~.

2.1.3. Validation as a vaccine strain

The vaccine derived from the MSV must be shown to be satisfactory with respect to safety and efficacy.

Even if pigs are not known for susceptibility to transmissible spongiform encephalopathy (TSE) agents, consideration should also be given to minimising the risk of TSE transmission by ensuring that animal origin materials from TSE-relevant species, if no alternatives exist for vaccine virus propagation, comply with the measures on minimising the risk of transmission of TSE.

Ideally, the vaccine virus in the final product should generally not differ by more than five passages from the master seed lot.

ASF vaccines should be presented in a suitable pharmaceutical form (e.g. lyophilisate or liquid form).

2.2. Method of manufacture

2.2.1. Procedure

The MLV ~~virus~~ is used to infect swine primary cell cultures obtained from specific-pathogen free pigs, the requirements for which are defined in specific monographs (Chapter 2.3.3 *Minimum requirements for the organisation and management of a vaccine manufacturing facility*, Section 2.4.2). Compared with primary cell cultures, use of a continuous cell line generally allows for more consistency, higher serial volumes in manufacturing and aligns better with a seed lot system. Thus, preferably a master cell bank based on an established, continuous cell line shown to support genetically stable ASFV replication and acceptable titres over several passages should be used.

Cell cultures shall comply with the requirements for cell cultures for production of veterinary vaccines in Chapter 1.1.8. Regardless of the production method, the substrate should be harvested under aseptic conditions and may be subjected to appropriate methods to release cell-associated virus (e.g. freeze–thaw cycles, detergent lysis). The harvest can be further processed by filtration and other purification methods. A stabiliser or other excipients may be added as appropriate. The vaccine is homogenised to ensure a uniform batch/serial.

2.2.2. Requirements for ingredients

All ingredients used for vaccine production should be in line with requirements in Chapter 1.1.8.

2.2.3. In-process controls

In-process controls will depend on the protocol of production: they include virus titration of bulk antigen and sterility tests.

2.2.4. Final product batch tests

i) Sterility

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

ii) Identity

Appropriate methods such as specific genome detection methods (e.g. specific differential real-time PCR) should be used for confirmation of the identity of the vaccine virus and differentiation from the parent strain of the virus as a potential contaminant.

iii) Purity

Appropriate methods should be used to ensure that the final product batch does not contain any residual wild-type ASFV.

iv) Safety

Batch safety testing is to be carried out unless consistent safety of the product is demonstrated and approved in the registration dossier and the production process is approved for consistency in accordance with the standard requirements referred to in chapter 1.1.8.

v) Batch/serial potency

Virus titration is a reliable indicator of vaccine potency once a relationship has been established between the vaccine minimum immunising dose (MID) (minimum protective dose) and titre of the modified live vaccine in vitro. In the absence of a demonstrated correlation between the virus titre and protection, an efficacy test will be necessary (Section C.2.3.3 *Efficacy requirements*, below).

vi) Residual humidity/residual moisture

The test should be carried out consistent with VICH[2] GL26 (*Biologicals: Testing of Residual Moisture*, 2003[3]). Required for MLV vaccines presented as lyophilisates ~~for suspension for injection~~.

2.3. Requirements for authorisation/registration/licensing

2.3.1. Manufacturing process

For regulatory approval of a vaccine, all relevant details concerning history of the pre-MSV, preparation of MSV, manufacture of the vaccine and quality control testing (Sections C.2.1 *Characteristics of the seed* and C.2.2 *Method of manufacture*) should be submitted to the authorities.

Information shall be provided from three preferably consecutive vaccine batches originating from the same MSV and representative of routine production~~, with a volume not less than 1/10, and more preferably with a volume not less than 1/3 of the typical industrial batch volume~~. The in-process controls are part of the manufacturing process.

[2] VICH: International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medical Products
[3] https://www.ema.europa.eu/en/documents/scientific-guideline/vich-gl26-biologicals-testing-residual-moisture-step-7_en.pdf

2.3.2. Safety requirements

For the purpose of gaining regulatory approval, the following safety tests should be performed satisfactorily.

As a minimum standard, vaccines should be tested for any pathogenic effects on healthy domestic pigs of the target age intended for use. Additional demonstration of MLV safety in breeding age gilts and pregnant sows is preferred ~~but not required as a minimum standard~~. If in the future a vaccine intended for use in breeding animals is developed, an evaluation of the impact of the vaccine on reproductive performance will be a standard safety requirement.

i) Safety in young animals

Carry out the test by each recommended route of administration using, in each case, piglets a minimum of ~~6~~ 4 ~~-~~weeks old and not older than 10-weeks old.

The test is conducted using no fewer than eight healthy piglets, and preferably no fewer than ten healthy piglets.

Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

Administer to each piglet a quantity of the vaccine virus equivalent to not less than ten times the maximum virus titre (e.g. 50% haemadsorption dose [HAD_{50}], 50% tissue culture infective dose [$TCID_{50}$]~~, quantitative PCR, etc.~~) (maximum release dose) likely to be contained in one dose of the vaccine.

To obtain individual and group mean baseline temperatures, the body temperature of each ~~vaccinated~~ piglet is measured on at least the 3 consecutive days preceding administration of the vaccine.

To confirm the presence or absence of fever accompanied by acute and chronic disease, observe the piglets 4 hours after vaccination and then at least once daily for at least 45 days, preferably 60 days post-vaccination. Carry out the daily observations for signs of acute and chronic disease using a quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.*, 2015a). These clinical signs should include fever, anorexia, recumbency, skin haemorrhage or cyanosis, joint swelling and necrotic lesions around the joints, respiratory distress and digestive findings).

At a minimum of 45 days post-vaccination, humanely euthanise all vaccinated piglets. Conduct gross pathology on spleen, lung, tonsil, and kidney tissue samples and at least three different lymph nodes (which should include lymph node closest to site of inoculation, gastrohepatic and submandibular nodes).

The vaccine complies with the test if:

- No piglet shows abnormal (local or systemic) reactions or notable signs of disease, or reaches the pre-determined humane endpoint defined in the clinical scoring system or dies from causes attributable to the vaccine;

- ~~The average body temperature increase for all vaccinated piglets (group mean) for the observation period does not exceed 1.5°C above baseline; and no individual piglet shows a temperature rise above baseline greater than 2.5°C for a period exceeding 3 days.~~

- On each day during the observation period, the maximum increase in body temperature above the baseline observed for each pig will be used to calculate the daily group mean temperature rise. This mean value should not

exceed 1.5°C and no individual pig should show a rise in temperature above baseline greater than 1.5°C for a period exceeding 3 consecutive days.

- No vaccinated pigs show notable signs of disease by gross pathology

ii) Safety test in pregnant sows and test for transplacental transmission

There is limited ~~currently an absence of~~ published information on ASFV pathogenesis in breeding-age gilts and in pregnant sows associated with ASFV transplacental infection and fetus abortion/stillbirth. If a label claim is pursued for use in breeding age gilts and sows, then a safety study in line with VICH GL44 (*Guidelines on Target Animal Safety for Veterinary Live and Inactivated Vaccines, Section 2.2. Reproductive Safety Test*, 2009[4]) should be completed.

iii) Horizontal transmission

The test is conducted using no fewer than 12 healthy piglets, a minimum of ~~6~~ 4-weeks old and not older than 10-weeks old and of the same origin, that do not have antibodies against ASFV, and blood samples are negative on real-time PCR. ~~All piglets are housed together from day 0 and the number of vaccinated animals is the same as the number of naïve, contact animals.~~ Co-mingle equal numbers of vaccinated and naïve, contact piglets from day 0 in the same pen or room.

Use vaccine virus at the least attenuated passage level that will be present between the master seed lot and a batch of the vaccine. Administer by each recommended route of administration to no fewer than six piglets a quantity of the vaccine virus equivalent to not less than the maximum virus titre (maximum release dose) likely to be contained in 1 dose of the vaccine.

To obtain individual and group mean baseline temperatures, the body temperature of each naïve, contact piglet is measured on at least the 3 consecutive days preceding co-mingling with vaccinated piglets. The body temperature of each naïve, contact piglet is then measured daily for at least 45 days, preferably 60 days.

To confirm the presence or absence of fever accompanied by disease, observe the naïve, contact piglets daily for at least 45 days, preferably 60 days. On each day during the observation period the maximum increase in body temperature above the baseline observed for each pig will be used to calculate the daily group mean temperature rise. This mean value should not exceed 1.5°C and no individual pig should show a rise in temperature above baseline greater than 1.5°C for a period exceeding 3 consecutive days. Carry out the daily observations for signs of acute and chronic clinical disease using a quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.*, 2015a). These clinical signs should include fever, anorexia, recumbency, skin haemorrhage or cyanosis, joint swelling and necrotic lesions around the joints, respiratory distress and digestive findings.

~~In addition,~~ Blood should be taken from the naïve contact piglets at least twice a week for the first 21 days post-vaccination and then on a weekly basis. From the blood samples, determine vaccine virus titres by quantitative virus isolation (HAD_{50}/ml, $TCID_{50}$/ml or other methods, e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above ~~infectious virus titres by quantitative virus isolation (e.g. HAD_{50}/ml or $TCID_{50}$/ml) and using a real-time PCR test~~.

[4] https://www.ema.europa.eu/en/documents/scientific-guideline/vich-gl44-target-animal-safety-veterinary-live-inactived-vaccines-step-7_en.pdf.

~~If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test only may be used.~~

Collect blood (serum) samples from the naïve contact pigs at least at day 21 and day 28 days and carry out an appropriate test to detect vaccine virus antibodies. At a minimum of 45 days, humanely euthanise all naïve, contact piglets. Conduct gross pathology on spleen, lung, tonsil, and kidney tissue samples and at least three different lymph nodes. Determine virus titres in all collected samples by quantitative virus isolation (HAD_{50}/mg or $TCID_{50}$/mg) or other appropriate methods (e.g. IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above ~~and real-time(RT)-PCR (see Section B.1. Identification of the agent). If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT detection) may be used~~.

The vaccine complies with the test if:

- No vaccinated or naïve contact piglet shows abnormal (local or systemic) reactions or notable signs of disease, reaches the predetermined humane endpoint defined in the clinical scoring system or dies from causes attributable to the vaccine;
- On each day during the observation period the maximum increase in body temperature above the baseline observed for each pig will be used to calculate the daily group mean temperature rise. This mean value should not exceed 1.5°C and no individual pig should show a rise in temperature above baseline greater than 1.5°C for a period exceeding 3 consecutive days ~~The average body temperature increase for all naïve, contact piglets (group mean) for the observation period does not exceed 1.5°C: above baseline; and no individual piglet shows a temperature rise above baseline greater than 2.5°C for a period exceeding 3 days~~;
- No naïve, contact piglet shows notable signs of disease by gross pathology ~~and no virus is detected in their blood or tissue samples~~;
- No or a low percentage of contact piglets test both real-time PCR positive and seropositive ~~No naïve contact pigs test positive for antibodies to the vaccine virus~~.

iv) Post-vaccination kinetics of viral replication (MLV blood and tissue dissemination) study

Prior to the reversion to virulence study (Section C2.3.2.v. below), a minimum of one study should be performed to determine the post-vaccination kinetics of virus replication in the blood (viremia), tissues and viral shedding.

The test consists of the administration of the vaccine virus from the master seed lot to no fewer than eight healthy piglets, and preferably ten healthy piglets, a minimum of ~~6~~ 4-weeks old and not older than 10-weeks old and of the same origin, that do not have antibodies against ASFV, and blood samples are negative on real-time PCR.

Administer to each piglet, using the recommended route of administration most likely to result in spread (such as the intramuscular route or intranasal route), a quantity of the master seed vaccine virus equivalent to not less than the maximum virus titre (maximum release dose) likely to be contained in 1 dose of the final product of the vaccine.

Record daily body temperatures and observe inoculated animals daily for clinical disease for at least 45 days, preferably 60 days.

Carry out the daily observations for signs of acute and chronic clinical disease using a quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.* (2015a). These clinical signs should include fever, anorexia, recumbency, skin haemorrhage or cyanosis, joint swelling and necrotic lesions around the joints, respiratory distress and digestive findings.

Collect blood samples from all the piglets at least two times per week from 3 days post-vaccination for the first 2 weeks, then weekly for the duration of the test. Determine vaccine virus titres by quantitative virus isolation (HAD_{50}/ml or $TCID_{50}$/ml) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples but results should be confirmed by infectious virus titration as described above ~~and using a real-time PCR test. If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test only may be used~~.

Determine which blood timepoint(s) should be used in the design of the reversion to virulence study (Section C2.3.2.v. below), for example, specific blood sample(s) at specific timepoints that show the highest titres should be considered for selection and use in the reversion to virulence study.

Collect oral, nasal and faecal swab samples (preferably devoid of blood to minimise assay interference) at least two times per week from 3-days post-vaccination for the first 2 weeks, then weekly for the duration of the test. Test the swabs for the presence of vaccine virus. Determine virus titres in all collected samples by quantitative virus isolation (HAD_{50}/ml or $TCID_{50}$/ml) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above ~~and using a real-time PCR test. If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT detection) may be used~~.

Euthanise at least two piglets on days 5, 7, 14, 21, and preferably on day 28 (±2 days at each timepoint) and collect spleen, lung, tonsil, kidney tissue samples and at least three different lymph nodes (which should include lymph node closest to site of inoculation, gastrohepatic and submandibular nodes). Determine virus titres in all collected samples by quantitative virus isolation (HAD_{50}/mg or $TCID_{50}$/mg) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above ~~and using real-time PCR test. If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT detection) may be used~~.

Determine which tissue(s) and timepoint(s) should be used to aid in the design of the reversion to virulence study (Section C.2.3.2.v), for example, specific tissues at specific timepoints which show the highest titres should be considered for selection and use in the reversion to virulence study.

v) Reversion to virulence

The test carried out should be consistent with VICH GL41 (Examination of live veterinary vaccines in target animals for absence of reversion to virulence, 2008[5]).

The test for increase in virulence consists of the administration of the vaccine master seed virus to healthy piglets of an age (e.g. between ~~6~~ 4 weeks and 10 weeks old) suitable for recovery of the strain and of the same origin, that do not

[5] https://www.ema.europa.eu/en/documents/scientific-guideline/vich-gl41-target-animal-safety-examination-live-veterinary-vaccines-target-animals-absence-reversion_en.pdf.

have antibodies against ASFV, and blood samples that are negative on real-time PCR. This protocol is typically repeated five times.

First passage (*p1*)

Administer to no fewer than two piglets, and preferably no fewer than four piglets using the intended route of administration for the final product, a quantity of the master seed vaccine virus equivalent to not less than the maximum virus titre (maximum release dose) likely to be contained in 1 dose of the final product of the vaccine. Observe inoculated animals daily for the appearance of ~~at least two and preferably at least three~~ clinical signs ~~and record daily body temperatures~~ using a quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.*, 2015a) and record daily body temperatures.

Based on results from at least one completed post-vaccination kinetics of viral replication (MLV ~~vaccine shed and spread (virus~~ blood and tissue dissemination study (Section C.2.3.2.iv above), collect an appropriate quantity of blood from each piglet on the predetermined ~~single~~ timepoint(s) (day ~~5~~ 3–13). Determine virus titres in individual blood samples by quantitative virus isolation (HAD_{50}/ml or $TCID_{50}$/ml) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above ~~and by real-time PCR. If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT detection) may be used~~. Identify the individual blood sample(s) with the highest infectious titre and reserve for the subsequent *in-vivo* passage (second pass, p2).

Based on results from at least one completed ~~vaccine virus~~ MLV blood and tissue ~~distribution~~ dissemination study (Section C.2.3.2.iv above), euthanise piglets on the predetermined timepoint (i.e. day 5, 7, 14, 21, or 28). Determine infectious virus titres in individual tissue samples by quantitative virus isolation (HAD_{50}/ml or $TCID_{50}$/ml) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above. ~~If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT detection) may be used.~~ Identify individual tissue sample type(s) with the highest infectious titre. Pool the tissues with the highest titres from different organs from ~~all~~ each animal~~s with the highest titres~~ and prepare ~~at least~~ a ~~10% virus~~ suspension to obtain a virus titre within the range used for inoculation in PBS, pH 7.2 kept at 4°C or at –70°C for longer storage.

Test each blood and tissue sample pool used for inoculation by PCR to confirm the absence of potential viral agent contaminants (i.e. CSFV, FMDV, PRRS, PCV2). Blood and pooled tissue (p1) are used to inoculate 2 ml of positive material diluted to the maximum release dose likely to be contained in 1 dose of the vaccine using the intended route of administration for the final product to each of at least two and ideally at least four further pigs of the same age and origin.

~~*Second pass* (*p2*)~~

If no virus is found at passage 1 (p1), repeat the administration by the intended route ~~once~~ again with the same pooled material (blood and pooled tissue, p1) in another ten healthy piglets of the same age and origin. If no virus is found at this point ~~during this second passage (p2) at this point~~, end the process here.

Second passage (p2)

If ~~however~~ virus is found in p1, carry out a second series of passages by administering 2 ml of positive material diluted to the maximum release dose likely to be contained in 1 dose of the vaccine using the intended route of administration for the final product to each of no fewer than two piglets, and preferably no fewer than four piglets of the same age and origin. Observe inoculated animals daily for the appearance of ~~at least two and preferably at least three~~ clinical signs using a quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.*, 2015a), ~~and~~ record daily body temperatures and determine infectious virus titres in individual blood and tissue samples as described for p1 above.

~~*Third and fourth pass (p3 and p4)*~~

If no virus is found at ~~in~~ (p2~~)~~, repeat the ~~intramuscular~~ administration by the intended route ~~once again~~ with the same pooled material (blood and pooled tissue, p2) in another eight healthy piglets of the same age and origin. If no virus is found at this point, end the process here.

Third and fourth passage (p3 and p4)

If~~, however,~~ virus is found on p2, carry out this passage operation no fewer than two additional times (p3 and p4) (to each of no fewer than two piglets, and preferably no fewer than four piglets of the same age and origin) and verifying the presence of the virus at each passage in blood and tissues. Observe inoculated animals daily for the appearance of ~~at least two and preferably at least three~~ clinical signs using a quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.*, 2015a) and record daily body temperatures.

Fifth passage (p5)

Administer 2 ml of the blood and pooled tissue (p4) to each of at least eight healthy piglets of the same age and origin. Observe inoculated animals daily for at least 28 days post-inoculation for the appearance of ~~at least two and preferably at least three~~ clinical signs using a quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.*, 2015a), ~~and~~ record daily body temperature and determine infectious virus titres in individual blood and tissue samples as described above.

The vaccine virus complies with the test if:

- No piglet shows abnormal (local or systemic) reactions, ~~or notable signs of disease, or~~ reaches the pre-determined humane endpoint defined in the clinical scoring system or dies from causes attributable to the vaccine; and
- There is no indication of increasing virulence (as monitored by daily body temperature accompanied by clinical sign observations) of the maximally passaged virus compared with the master seed virus.

At a minimum, a safe MLV vaccine shall demonstrate ALL the following features (minimal standards):

- Absence of fever (on each day during the observation period, the maximum increase in body temperature above the baseline observed for each pig will be used to calculate the daily group mean temperature rise. This mean value should not exceed 1.5°C and no individual pig should show a rise in temperature above baseline greater than 1.5°C ~~(defined as average body temperature increase for all vaccinated piglets (group mean) for the observation period does not exceed 1.5°C above baseline; and no individual~~

~~piglet shows a temperature rise above baseline greater than 2.5°C~~ for a period exceeding 3 days);

- Absence of chronic and acute clinical signs and gross pathology over the entire test period or ~~minimal chronic~~ mild clinical signs (~~defined as~~ e.g. mild swollen joints with a low clinical score that resolve within 1 week).
- Minimal (defined as no naïve, contact piglet shows notable signs of disease by clinical signs and gross pathology and no or a low percentage of contact piglets test both real-time PCR positive and seropositive) or no vaccine virus transmission (defined as no naïve, contact piglet shows notable signs of disease by clinical signs and gross pathology and no contact piglets test both real-time PCR positive and seropositive) over the entire test period;
- Absence of an increase in virulence (genetic and phenotypic stability) (complies with the reversion to virulence test).

In addition, for regulatory approval, ASF MLV ~~the~~ vaccines ~~in their commercial presentation before being authorised for general use~~ should be tested for safety ~~in the~~ under field conditions (see chapter 1.1.8 Section 7.2.3). ~~Additional~~ Field safety studies generally ~~evaluation studies~~ may include measurement of body temperatures, observation of local or systemic reactions and, where appropriate, performance measurements ~~but are not limited to: environmental persistence (e.g. determination of virus recovery from bedding or other surfaces), assessment of immunosuppression, and negative impacts on performance~~.

2.3.3. Efficacy requirements

i) Protective dose

Vaccine efficacy is estimated in immunised animals directly, by evaluating their resistance to live virus challenge. The test consists of a vaccination/challenge trial in piglets a minimum of ~~6~~ 4-weeks old and not more than 10-weeks old, free of antibodies to ASFV, and negative blood samples by real-time PCR. The test is conducted using no fewer than 15 and preferably no fewer than 24 vaccinated pigs, and no fewer than five non-vaccinated control piglets.

The test is conducted to determine the minimal immunising dose (MID) (also referred to as the minimal protective dose [MPD] or protective fraction); using at least three groups of no fewer than five and preferably not fewer than eight vaccinated piglets per group, and one additional group of no fewer than five non-vaccinated piglets of the same age and origin as controls. Use vaccine containing virus at the highest passage level that will be present in a batch of vaccine.

Each group of piglets, except the control group, is immunised with a different vaccine virus content in the same vaccine volume. In at least one vaccinated group, piglets are immunised with a vaccine dose containing not more than the minimum virus titre (minimum release dose) likely to be contained in one dose of the vaccine as stated on the label.

Twenty-eight days (±2 days) after the single ~~injection~~ dose of vaccine (or if using two ~~injections~~ doses of the vaccine then 28 days [±2 days] following the second ~~injection~~ dose), challenge all the piglets by the intramuscular route. If previous studies have demonstrated acceptable efficacy using IM challenge, then a different challenge route (e.g. direct contact, oral or oronasal) may be used. Challenged, vaccinated piglets may be housed in one or more separate pens in the same room or in different rooms. Challenged, naïve controls can be housed in one or more rooms that are separate from challenged, vaccinated piglets.

Carry out the test using an ASFV representative strain of the epidemiologically relevant field strain(s) where the vaccine is intended for use (e.g. ASFV B646L [p72] genotype II pandemic strain and other p72 virulent genotype of recognised

epidemiologic importance). For gene deleted, recombinant MLV viruses, if neither challenge virus type is available, then carry out the test with the parental, virulent virus used to generate the MLV recombinant virus. Use a 10e3–10e4 HAD_{50} (or $TCID_{50}$ for non-HAD viruses) challenge dose sufficient to cause death or meet the humane endpoint in 100% of the nonvaccinated piglets in less than 21 days. Higher or lower challenge doses can be considered if appropriately justified.

The rectal temperature of each vaccinated piglet is measured on at least the 3 days preceding administration of the challenge virus, at the time of challenge, 4 hours after challenge, and then daily for the observation period of at least ~~28~~ 45 days, preferably ~~35~~ 60 days. ~~Observe the piglets at least daily for at least 28 days, preferably 35 days.~~ Carry out the daily observations for signs of acute and chronic clinical disease using a quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.*, 2015). These clinical signs should include fever, anorexia, recumbency, skin haemorrhage or cyanosis, joint swelling and necrotic lesions around the joints, respiratory distress and digestive findings.

Collect oral, nasal, anal and blood samples from the vaccinated challenged piglets at least two times ~~once~~ per week from 3 days post-challenge for at least ~~28~~ 14 days, then weekly up to 35 days post-challenge and then every 14 days up to the end of the observation period ~~preferably 35 days~~. From the blood samples, determine infectious virus titres by quantitative virus isolation (HAD_{50}/ml or $TCID_{50}$/ml) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above ~~and using a real-time PCR test. If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test only may be used~~.

At the end of the test period, humanely euthanise all vaccinated challenged piglets. Conduct gross pathology (and histopathology if considered necessary) on spleen, lung, tonsil, and kidney tissue samples and at least three different lymph nodes (which should include lymph node closest to site of inoculation, gastrohepatic and submandibular nodes). Determine virus titres in all collected samples by quantitative virus isolation (HAD_{50}/mg or $TCID_{50}$/mg) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above ~~and real-time PCR (see Section B.1. Identification of the agent). If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT detection) may be used~~.

The test is invalid if ~~fewer than 100%~~ the difference between ~~in~~ the number of unvaccinated control piglets infected with the live challenge virus and the number of vaccinated / challenged piglets ~~vaccinated with the minimum release dose~~ that die or reach a humane endpoint is not statistically significant.

The vaccine (or a specific vaccine virus dose if conducting a vaccine dose titration study) complies with the test if:

- No vaccinated challenged piglet dies or ~~shows abnormal (local or systemic) reactions,~~ reaches the humane endpoint ~~or dies~~ from causes attributable to ASF;
- On each day during the observation period the maximum increase in body temperature above the baseline observed for each pig will be used to calculate the daily group mean. This mean value should not exceed 1.5°C and no individual pig should show a rise in temperature above baseline greater than 2.0°C for a period exceeding 2 consecutive days T~~he average body temperature increase for all vaccinated challenged piglets (group mean) for the observation period does not exceed 2.0°C above baseline; and no individual piglet shows a temperature rise above baseline greater than 2.0°C~~;

- The vaccinated challenged piglets display a reduction or absence of typical acute clinical signs of disease and gross pathology and a reduction or absence of challenge virus levels in blood and tissues.

ii) Assessment for horizontal transmission (challenge virus shed and spread study)

The ASF basic reproduction number, R0, can be defined as the average number of secondary ASF disease cases caused by a single ASFV infectious pig during its entire infectious period in a fully susceptible population (Hayes *et al*., 2021). In general, if the ASFV effective reproduction number Re=R0 × (S/N) (S= susceptible pigs; N= total number of pigs in a given population) is greater than 1.0, disease is predicted to spread. Ideally, ASF vaccination should reduce Re to less than 1.0 by reducing the number of susceptible, naïve, contact pigs exposed to vaccinated, infected pigs.

To evaluate ASF vaccine impact on ASF disease transmission, the test consists of a vaccination/challenge trial in piglets a minimum of ~~6~~ 4-weeks old and not older than 10-weeks old, free of antibodies to ASFV, and negative blood samples by real-time PCR.

The test is conducted using no fewer than 15 healthy piglets at a ratio comprising twice the number of vaccinated piglets to naïve piglets (e.g. ten vaccinated and five naïve). Use vaccine containing virus at the highest passage level that will be present in a batch of the vaccine.

The quantity of vaccine virus administered to each pig is equivalent to be not less than the minimum virus titre (minimum dose) likely to be contained in one dose of the vaccine as stated on the label. Following immunisation, vaccinated and naïve piglets should continue to be co-mingled.

Twenty-eight days (±2 days) after the single ~~injection~~ dose of vaccine (or if using two ~~injections~~ doses of the vaccine then 28 days [±2 days] following the second ~~injection~~ dose), temporarily separate [into different pen(s) or room(s)] all vaccinated piglets from naïve piglets. Challenge all vaccinated piglets by the intramuscular or other previously verified route. Carry out the challenge using an ASFV representative strain of the epidemiologically relevant field strain(s) where the vaccine is intended for use (e.g. ASFV B646L [p72] genotype II pandemic strain and other p72 virulent genotype of recognised epidemiological importance). For gene deleted, recombinant MLV viruses, if neither challenge virus type is available, then carry out the test with the parental, virulent virus used to generate the MLV recombinant virus. Use a 10e3–10e4 HAD_{50} (or $TCID_{50}$ for non-HAD viruses challenge dose sufficient to cause death or met the humane endpoint in 100% of the nonvaccinated piglets in less than 21 days. Higher or lower challenge doses can be considered if appropriately justified.

Approximately 18–24 hours later, re-introduce naïve piglets to vaccinated, challenged piglets and allow for direct nose to nose contact exposure with vaccinated, challenged piglets. Allow for continuous contact exposure by co-mingling both groups through the end of the study. If more than one pen or room is used for co-housing, following reintroduction initially maintain a ratio of 2:1 of challenged, vaccinated piglets to contact exposed, naïve piglets.

The rectal temperature of each contact piglet is measured on at least the 3 days preceding administration of the challenge virus to vaccinated pigs, immediately prior to direct contact exposure, 4 hours post-contact exposure, and then daily for at least 28~~, preferably 35~~ days and twice a week for at least 60 days. Observe all contact exposed piglets ~~at least~~ daily for at least 28 days, and then twice a week for at least 60 days ~~preferably for at least 35 days~~.

Carry out the ~~daily~~ observations in each contact piglet for signs of acute and chronic clinical disease using a quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.,* 2015a). These clinical signs should include fever, anorexia, recumbency, skin haemorrhage or cyanosis, joint swelling and necrotic lesions around the joints, respiratory distress and digestive findings.

In addition, ~~blood should be taken from the naïve contact piglets at least twice a week from 3 days post-contact exposure for the duration~~ collect blood samples from the contact piglets at least two times per week from 3 days post-contact for at least 14 days, then weekly up to 35 days post-contact exposure and then every 14 days up to the end of the test period. Determine virus titres in all collected samples by quantitative virus isolation (HAD_{50}/mg or $TCID_{50}$/mg) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above ~~From the blood samples, determine infectious challenge virus titres by quantitative virus isolation (HAD_{50}/ml or $TCID_{50}$/ml) and using a real-time PCR test. If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test only may be used~~.

Collect blood (serum) samples from the naïve contact pigs at least at day 21 and day 28 (±2 days), and at the end of the test period, and carry out an appropriate test to detect vaccine virus antibodies.

Collect oral, nasal and faecal swab samples (preferably devoid of blood to minimise assay interference) from all contact-exposed naïve piglets at least two times per week from 3-days post-contact exposure for the first 2 weeks, then weekly for the duration of the test and test swabs for the presence of challenge virus. Determine virus titres in all collected samples by quantitative virus isolation (HAD_{50}/mg or $TCID_{50}$/mg) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above ~~Determine virus titres in all collected samples by quantitative virus isolation (HAD_{50}/ml or $TCID_{50}$/ml) and using a real-time PCR test. If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT detection) may be used~~.

At the end of the test period, humanely euthanise all contact piglets. Conduct gross pathology on spleen, lung, tonsil, and kidney tissue samples and at least three different lymph nodes. (which should include lymph node closest to site of inoculation, gastrohepatic and submandibular nodes). Determine virus titres in all collected samples by quantitative virus isolation (HAD_{50}/mg or $TCID_{50}$/mg) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above ~~Determine virus titres in all collected samples by quantitative virus isolation (HAD_{50}/mg or $TCID_{50}$/mg) and real-time PCR (see Section B.1. Identification of the agent). If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT detection) may be used~~.

The test is invalid if the vaccine fails to comply with the compliance criteria described for the protected dose test in vaccinated pigs (Section C.2.3.3.i above).

If the manufacturer claims that the vaccine induces sterilising immunity, the vaccine complies with the test for a reduction in horizontal disease transmission if all the following conditions are satisfied:

- No naïve, contact exposed piglet shows abnormal (local or systemic) reactions, reaches the defined humane endpoint or dies from causes attributable to ASF;

- No naïve, contact exposed piglet displays fever accompanied by typical signs of disease, including gross pathology.
- Naïve contact pigs show an absence of challenge virus in blood and tissues.
- No naïve contact pigs test positive for antibodies to the challenge virus.

Otherwise, the vaccine complies with the test for a reduction in horizontal disease transmission if:

- Naïve contact pigs show a reduction or absence of challenge virus levels in blood and tissues.
- None of or a reduced number of naïve contact exposed pigs test positive for antibodies to the challenge virus.

At a minimum, an efficacious MLV vaccine shall demonstrate ALL the following features (minimal standards):

- Protects against mortality;
- Reduces acute disease (fever accompanied by a reduction of typical clinical and pathological signs of acute disease)
- Reduces levels of viral shedding and viraemia.
- Reduces horizontal disease transmission (~~no~~ none of or a reduced number of naïve, contact exposed piglets shows abnormal [local or systemic] reactions, reaches the humane endpoint or dies from causes attributable to ASF, ~~and~~ displays fever accompanied by typical acute disease signs caused by ASF) and test positive for antibodies to the challenge virus.
- ~~Reduces levels of viral shedding and viraemia.~~

In general, for regulatory approval, ASF MLV ~~addition, the~~ vaccines ~~in their commercial presentation before being authorised for general use~~ should be tested for efficacy ~~in the~~ under field conditions (see chapter 1.1.8 Section 7.2.3). ~~Additional~~ Field efficacy ~~evaluation~~ studies ~~may~~ generally include ~~but are not limited to: onset of immunity, duration of immunity, and impact on disease transmission~~ measurement of relevant efficacy parameters including but limited to mortality, clinical signs, impact on disease transmission, performance parameters.

2.3.4. Duration of immunity

Although not included in the guidance for ASF MLV first generation vaccines, manufacturers are ~~encouraged~~ required, as part of the authorisation procedure, to define and demonstrate the duration of immunity of a given vaccine ~~by evaluation of potency at the end of the claimed period of protection~~.

2.3.5. Stability

Stability of the vaccine should be demonstrated over the shelf life recommended for the product. Although not included in the standards for first generation MLV ASF vaccines, manufacturers are ~~encouraged~~ required, as part of the authorisation procedure, to generate data supporting the retention of immunogenicity over a defined period of ~~validity~~ time of a lyophilised or other pharmaceutical form of the ASF vaccine as part of the authorisation procedure.

REFERENCES

ACHENBACH J.E., GALLARDO C., NIETO-PELEGRÍN E., RIVERA-ARROYO B., DEGEFA-NEGI T., ARIAS M., JENBERIE S., MULISA D.D., GIZAW D., GELAYE E., CHIBSSA T.R., BELAYE A., LOITSCH A., FORSA M., YAMI M., DIALLO A., SOLER A., LAMIEN C.E. & SÁNCHEZ-VIZCAÍNO J.M. (2017). Identification of a New Genotype of African Swine Fever Virus in Domestic Pigs from Ethiopia. *Transbound. Emerg. Dis*., **64**, 1393–1404.

AGÜERO M., FERNÁNDEZ J., ROMERO L., SANCHEZ C., ARIAS M. & SÁNCHEZ-VIZCAÍNO J.M. (2003). Highly sensitive PCR assay for the routine diagnosis of African swine fever virus in clinical samples, *J. Clin. Microbiol*., **41**, 4431–4434.

AGÜERO M., FERNÁNDEZ J., ROMERO L., ZAMORA M.J., SANCHEZ C., BELÁK S., ARIAS M. & SÁNCHEZ-VIZCAÍNO J.M. (2004). A highly sensitive and specific gel-based multiplex RT-PCR assay for the simultaneous and differential diagnosis of African swine fever and Classical swine fever. *Vet. Res*., **35**, 1–13.

ALEJO A., MATAMOROS T., GUERRA M. & ANDRÉS G. (2018). A Proteomic Atlas of the African Swine Fever Virus Particle. *J. Virol*., **92**, pii: e01293-18. doi: 10.1128/JVI.01293-18.

BARASONA J.A., GALLARDO C., CADENAS-FERNÁNDEZ E., JURADO C., RIVERA B., RODRÍGUEZ-BERTOS A., ARIAS M. & SÁNCHEZ-VIZCAÍNO J.M. (2019). First oral vaccination of Eurasian wild boar against African swine fever virus genotype II. *Front. Vet. Sci.,* **6**, 137. https://doi: 10.3389/fvets.2019.00137.

BASTO A.P., PORTUGAL R.S., NIX R.J., CARTAXEIRO C., BOINAS F., DIXON L.K., LEITAO A. & MARTINS C. (2006). Development of a nested PCR and its internal control for the detection of African swine fever virus (ASFV) in *Ornithodoros erraticus*. *Arch. Virol.,* **151**, 819–826

BISHOP R.P., FLEISCHAUER C., DE VILLIERS E.P., OKOTH E.A., ARIAS M., GALLARDO C. & UPTON C. (2015). Comparative analysis of the complete genome sequences of Kenyan African swine fever virus isolates within p72 genotypes IX and X. *Virus Genes*, **50**, 303–309.

BORCA M.V., RAI A., RAMIREZ-MEDINA E., SILVA E., VELAZQUEZ-SALINAS L., VUONO E., PRUITT S., ESPINOZA N. & GLADUE D.P. (2021). A Cell Culture-Adapted Vaccine Virus against the Current African Swine Fever Virus Pandemic Strain. *J. Virol.,* **95**, e0012321. https://doi: 10.1128/JVI.00123-21.

BOSHOFF C.I., BASTOS A.D., GERBER L.J. & VOSLOO W. (2007). Genetic characterisation of African swine fever viruses from outbreaks in southern Africa (1973–1999). *Vet. Microbiol.,* **121**, 45–55.

BOURRY O., HUTET E., LE DIMNA M., LUCAS P., BLANCHARD Y., CHASTAGNER A., PABOEUF F. & LE POTIER M.F. (2022).Oronasal or Intramuscular Immunization with a Thermo-Attenuated ASFV Strain Provides Full Clinical Protection against Georgia 2007/1 Challenge. *Viruses*,**14**, 2777. doi: 10.3390/v14122777

BRAKE D.A. (2022). African Swine Fever Modified Live Vaccine Candidates: Transitioning from Discovery to Product Development through Harmonized Standards and Guidelines. *Viruses*, 14, 2619. doi: 10.3390/v14122619

CADENAS-FERNÁNDEZ E., SÁNCHEZ-VIZCAÍNO J.M., KOSOWSKA A., RIVERA B., MAYORAL-ALEGRE F., RODRÍGUEZ-BERTOS A., YAO J., BRAY J., LOKHANDWALA S., MWANGI W. & BARASONA J.A. (2020). Adenovirus-vectored African Swine Fever Virus Antigens Cocktail Is Not Protective against Virulent Arm07 Isolate in Eurasian Wild Boar. *Pathogens*, **9**, 171. doi: 10.3390/pathogens9030171.

CHAPMAN D.A., DARBY A.C., DA SILVA M., UPTON C., RADFORD A.D. & DIXON L.K. (2011). Genomic analysis of highly virulent Georgia 2007/1 isolate of African swine fever virus. *Emerg. Infect. Dis*, **17**, 599–605.

CHEN W., ZHAO D., HE X., LIU R., WANG Z., ZHANG X., LI F., SHAN D., CHEN H., ZHANG J., WANG L., WEN Z., WANG X., GUAN Y., LIU J. & BU Z. (2020).A seven-gene-deleted African swine fever virus is safe and effective as a live attenuated vaccine in pigs. Sci. *China Life Sci.,* **63**, 623. https://doi: 10.1007/s11427-020-1657-9.

COSTARD S., MUR L., LUBROTH J., SANCHEZ-VIZCAINO J.M. & PFEIFFER D.U. (2013). Epidemiology of African swine fever virus. *Virus Res*., **173**, 191–197.

DE LEÓN P., BUSTOS M.J. & CARRASCOSA A.L. (2013). Laboratory methods to study African swine fever virus. *Virus Res*., **173**, 168–179.

DE VILLIER E.P., GALLARDO C., ARIAS M., DA SILVA M., UPTON C., MARTIN R. & BISHOP R.P. (2010). Phylogenomic analysis of 11 complete African swine fever virus genome sequences. *Virology*, **400**, 128–136.

DIXON L.K., ESCRIBANO J.M., MARTINS C., ROCK D.L., SALAS M.L. & WILKINSON P.J. (2005). *In:* Virus Taxonomy, VIIIth Report of the ICTV, Fauquet C.M., Mayo M.A., Maniloff J, Desselberger U.& Ball L.A., eds. Elsevier/Academic Press, London, UK, 135–143.

ESCRIBANO J.M., PASTOR M.J. & SANCHEZ VIZCAINO J.M. (1989). Antibodies to bovine serum albumin in swine sera: implications for false positive reactions in the sero diagnosis of African swine fever. *Am. J. Vet. Res.,* **50**, 1118–1122.

FERNÁNDEZ-PACHECO P., NIETO R., SIMÓN A., GARCÍA CASTEY T.A., MARTÍN E., ARIAS M. & GALLARDO C. (2016). Comparative evaluation of the performance of six ELISA tests for the detection of antibodies against African swine fever virus (ASFV). EPIZONE 10th Annual Meeting, Madrid, Spain, 27–29 September 2016, p. https://www.epizone-eu.net/en/Home/Downloads.htm

FERNÁNDEZ-PINERO J., GALLARDO C., ELIZALDE M., RASMUSSEN T.B., STAHL K., LOEFFEN W., BLOME S., BATTEN C., CROOKE H., LE POTIER M.F., UTTENTHAL Å., LEBLANC N., ALBINA E., KOWALCZYK A., MARKOWSKA-DANIEL I., TIGNON M., DE MIA G.M., GIAMMARIOLI M., ARIAS M. & HOFFMANN B. (2010). EPIZONE ring trial on ASFV real-time PCR. Annual Meeting of National African swine fever Laboratories, 18 May 2010, Pulawy, Poland.

FERNÁNDEZ-PINERO J., GALLARDO C., ELIZALDE M., ROBLES A., GÓMEZ C., BISHOP R., HEATH L., COUACY-HYMANN E., FASINA F.O., PELAYO V., SOLER A & ARIAS M. (2013). Molecular diagnosis of African Swine Fever by a new real-time PCR using universal probe library. *Transbound. Emerg. Dis*., **60**, 48–58.

FORTH J.H., CALVELAGE S., FISCHER M., HELLERT J., SEHL-EWERT J., ROSZYK H., DEUTSCHMANN P., REICHOLD A., LANGE M., THULKE H.-H., SAUTER-LOUIS C., HÖPER D., MANDYHRA S., SAPACHOVA M., BEER M. & BLOME S. (2023). African swine fever virus - variants on the rise. *Emerg. Microbes infect.*, **12**, 2146537.

GALLARDO C., FERNÁNDEZ-PINERO J., PELAYO V., GAZAEV I., MARKOWSKA-DANIEL I., PRIDOTKAS G., NIETO R., FERNÁNDEZ-PACHECO P., BOKHAN S., NEVOLKO O., DROZHZHE Z., PÉREZ C., SOLER A., KOLVASOV D. & ARIAS M. (2014). Genetic variation among African swine fever genotype II viruses, eastern and central Europe. *Emerg. Infect. Dis.*, **20**, 1544–1547.

GALLARDO C., MWAENGO D.M., MACHARIA J.M., ARIAS M., TARACHA E.A., SOLER A., OKOTH E., MARTÍN E., KASITI J. & BISHOP RP. (2009). Enhanced discrimination of African swine fever virus isolates through nucleotide sequencing of the p54, p72, and pB602L (CVR) genes. *Virus Genes*, **38** 85–95.

GALLARDO C., NIETO R., SOLER A., PELAYO V., FERNÁNDEZ-PINERO J., MARKOWSKA-DANIEL I., PRIDOTKAS G., NURMOJA I., GRANTA R., SIMÓN A., PÉREZ C., MARTÍN E., FERNÁNDEZ-PACHECO P. & ARIAS M. (2015b). Assessment of African swine fever diagnostic techniques as a response to the epidemic outbreaks in Eastern European Union countries: How to improve surveillance and control programs. *J. Clin. Microbiol*., **53**, 2555–2565.

GALLARDO C., SOLER A., NIETO R., CARRASCOSA A.L., DE MIA G.M., BISHOP R.P., MARTINS C., FASINA F.O., COUACY-HYMMAN E., HEATH L., PELAYO V., MARTIN E., SIMON A., MARTIN R., OKURUT A.R., LEKOLOL I., OKOTH E. & ARIAS M. (2013). Comparative evaluation of novel African swine fever virus (ASF) antibody detection techniques derived from specific ASF viral genotypes with the OIE internationally prescribed serological tests. *Vet. Microbiol*., **162**, 32–43.

92 GALLARDO C., SOLER A., NIETO R., SÁNCHEZ M.A.S., MARTINS E., PELAYO V., CARRASCOSA A., REVILLA Y.,
93 SIMON A., BRIONES V., SÁNCHEZ-VIZCAÍNO J.M. & ARIAS M. (2015a). Experimental Transmission of
94 African Swine Fever (ASF) Low Virulent Isolate NH/P68 by Surviving Pigs. *Transbound. Emerg. Dis*.,
95 **62**, 612–622.

96 GLADUE D.P. & BORCA M.V. (2022).Recombinant ASF Live Attenuated Virus Strains as Experimental
97 Vaccine Candidates. *Viruses*, **14**, 878. http://doi:10.3390/v14050878.

98 GLADUE D.P., RAMIREZ-MEDINA E,. VUONO E., SILVA E., RAI A., PRUITT S., ESPINOZA N., VELAZQUEZ-
99 SALINAS L. & BORCA M.V. (2021). Deletion of the A137R Gene from the Pandemic Strain of African
100 Swine Fever Virus Attenuates the Strain and Offers Protection against the Virulent Pandemic Virus. *J.*
101 *Virol.,* **95** (21):e0113921. doi: 10.1128/JVI.01139-21. Epub 2021 Aug 18. PMID: 34406865; PMCID:
102 PMC8513468.

103 HAINES F.J., HOFMANN M.A., KING D.P., DREW T.W. & CROOKE H.R. (2013). Development and validation
104 of a multiplex, real-time RT PCR assay for the simultaneous detection of classical and African swine
105 fever viruses. *PLoS One*, **8** (7).

106 HAYES B.H., ANDRAUD M., SALAZAR L.G., ROSE N. & VERGNE T. (2021). Mechanistic modelling of African
107 swine fever: A systematic review. *Prev. Vet. Med*., **191**, 105358. doi:
108 10.1016/j.prevetmed.2021.105358.

109 KITAMURA T., MASUJIN K., YAMAZOE R., KAMEYAMA K.I., WATANABE M., IKEZAWA M., YAMADA M. & KOKUHO
110 T. (2023). A Spontaneously Occurring African Swine Fever Virus with 11 Gene Deletions Partially
111 Protects Pigs Challenged with the Parental Strain. *Viruses*, **15**, 311. doi: 10.3390/v15020311. PMID:
112 36851524; PMCID: PMC9966947.

113 KING K., CHAPMAN D., ARGILAGUET J.M., FISHBOURNE E., HUTET E., CARIOLET R., HUTCHINGS G., OURA
114 C.A., NETHERTON C., MOFFAT K., TAYLOR G., LE POTIER M.-F., DIXON L.K. & TAKAMATSU H.-H. (2011).
115 Protection of European domestic pigs from virulent African isolates of African swine fever virus by
116 experimental immunisation. Vaccine, 29, 4593–4600. https://doi: 10.1016/j.vaccine.2011.04.052

117 KING D.P., REID S.M., HUTCHINGS G.H., GRIERSON S.S., WILKINSON P.J., DIXON L.K., BASTOS A.D.S. &
118 DREW T.W. (2003). Development of a TaqMan® PCR assay with internal amplification control for the
119 detection of African swine fever virus. *J. Virol. Methods*, **107**, 53–61.

120 LIU Y., XIE Z., LI Y., SONG Y., DI D., LIU J., GONG L., CHEN Z., WU J., YE Z., LIU J., YU W., LV L., ZHONG Q.,
121 TIAN C., SONG Q., WANG H. & CHEN H. (2023). Evaluation of an I177L gene-based five-gene-deleted
122 African swine fever virus as a live attenuated vaccine in pigs. *Emerg. Microbes Infect*., **12**, 2148560.
123 doi: 10.1080/22221751.2022.2148560. PMID: 36378022; PMCID: PMC9769145.

124 LUBISI B.A., BASTOS A.D., DWARKA R.M. & VOSLOO W. (2005). Molecular epidemiology of African swine
125 fever in East Africa. *Arch Virol.,* **150**, 2439–2452.

126 MASUJIN K., KITAMURA K., KAMEYAMA K.I., OKADERA K., NISHI T., TAKENOUCHI T., KITANI H. & KOKUHO T.
127 (2021). An immortalized porcine macrophage cell line competent for the isolation of African swine fever
128 virus. *Sci. Rep.,* **11**, 4759. doi.org/10.1038/s41598-021-84237-2.

129 MONTEAGUDO P.L., LACASTA A., LOPEZ L., BOSCH J., COLLADO J., PINA-PEDRERO S., CORREA-FIZ F.,
130 ACCENSI F., NAVAS M.J., VIDAL E., BUSTOS M.J., RODRÍGUEZ J.M., GALLEI A., NIKOLIN V., SALAS M.L. &
131 RODRÍGUEZ F. (2017). BA71deltaCD2: A new recombinant live attenuated African swine fever virus with
132 cross-protective capabilities. *J. Virol.*, **91**, e1058–e1117. https://doi: 10.1128/JVI.01058-17.

133 NCBI (NATIONAL CENTER FOR BIOTECHNOLOGY INFORMATION) (2020).
134 https://www.ncbi.nlm.nih.gov/nuccore/FR682468. Accessed 4/4/2023.

135 NIX R.J., GALLARDO C., HUTCHINGS G., BLANCO E. & DIXON L.K. (2006). Molecular epidemiology of African
136 swine fever virus studied by analysis of four variable genome regions. *Arch. Virol*., **151**, 2475–244.

137 O'DONNELL V., HOLINKA L.G., GLADUE, DP., SANFORD B., KRUG P.W., LU X., ARZT J., REESE B., CARRILLO
138 C., RISATTI G.R. & BORCA M.V. (2015). African swine fever virus Georgia isolate harboring deletions of
139 MGF360 and MGF505 genes is attenuated in swine and confers protection against challenge with
140 virulent parental virus. *J. Virol.*, **89**, 6048. https://doi: 10.1128/JVI.00554-15.

141 O'DONNELL V., RISATTI G.R., HOLINKA L.G., KRUG P.W., CARLSON J., VELAZQUEZ-SALINAS L., AZZINARO
142 P.A., GLADUE D.P. & BORCA M.V. (2016). Simultaneous Deletion of the 9GL and UK Genes from the
143 African Swine Fever Virus Georgia 2007 Isolate Offers In-creased Safety and Protection against
144 Homologous Challenge. *J. Virol.*, **91**, e01760-16. https://doi: 10.1128/JVI.01760-16.

145 PASTOR M.J., ARIAS M. & ESCRIBANO J.M. (1990). Comparison of two antigens for use in an enzyme-
146 linked immunosorbent assay to detect African swine fever antibody. *Am. J. Vet. Res.,***51**, 1540–1543.

147 PASTOR M.J., LAVIADA M.D., SANCHEZ VIZCAINO J.M. & ESCRIBANO J.M. (1989). Detection of African swine
148 fever virus antibodies by immunoblotting assay. *Can. J. Vet. Res.,* **53**, 105–107.

149 PENRITH M.L., VAN HEERDEN J., HEATH L., ABWORO E.O. & BASTOS A.D.S. (2022). Review of the Pig-
150 Adapted African Swine Fever Viruses in and Outside Africa. *Pathogens*, **11**, 1190. doi:
151 10.3390/pathogens11101190.

152 PÉREZ-NÚÑEZ D., SUNWOO S.-Y., GARCÍA-BELMONTE R., KIM C., VIGARA-ASTILLERO G., RIERA E., KIM D.-
153 M., JEONG J., TARK D., KO Y.-S., YOU Y.-K. & REVILLA Y. (2022). Recombinant African Swine Fever Virus
154 Arm/07/CBM/c2 Lacking CD2v and A238L Is Attenuated and Protects Pigs against Virulent Korean Paju
155 Strain. *Vaccines*, **10**, 1992. https://doi.org/10.3390/vaccines10121992.

156 PORTUGAL R., COELHO J., HÖPER D., LITTLE N.S., SMITHSON C., UPTON C., MARTINS C., LEITÃO A. & KEIL
157 G.M. (2015). Related strains of African swine fever virus with different virulence: genome comparison
158 and analysis. *J. Gen. Virol.*, **96** (Pt 2), 408–419.

159 PORTUGAL R., GOATLEY L.C., HUSMANN R., ZUCKERMANN F.A. & DIXON L.K. (2020). A porcine
160 macrophage cell line that supports high levels of replication of OURT88/3, an attenuated strain of
161 African swine fever virus. *Emerg. Microbes Infect.*, **9**, 1245–1253.
162 doi.org/10.1080/22221751.2020.1772675.

163 QUEMBO C.J., JORI F., VOSLOO W. & HEATH L. (2018). Genetic characterization of African swine fever
164 virus isolates from soft ticks at the wildlife/domestic interface in Mozambique and identification of a
165 novel genotype. *Transbound. Emerg. Dis.*, **65**, 420–431.

166 SÁNCHEZ-VIZCAÍNO J.M. (1987). African swine fever diagnosis. *In:* African Swine Fever, Becker Y., ed.
167 Martinus Nijhoff, Boston, USA, 63–71.

168 SÁNCHEZ-VIZCAÍNO J.M. & ARIAS M. (2012). African swine fever. *In*: Diseases of Swine, tenth Edition,
169 Straw B., D'Allaire S., Mengeling W., Taylor D., eds. Iowa State University, USA, pp.396–404.

170 SÁNCHEZ-VIZCAÍNO J.M., MARTINEZ-LÓPEZ B., MARTINEZ-AVILÉS M., MARTINS C., BOINAS B., VIAL L.,
171 MICHAUD V., JORI F., ETTER E., ALBINA E. & ROGER F. (2009). Scientific Review on African swine fever.
172 CFP/EFSA/AHAW/2007/02, pp: 1–141.

173 SÁNCHEZ-VIZCAÍNO J.M., MUR L., GOMEZ-VILLAMANDOS J.C. & CARRASCO L. (2015). An update on the
174 epidemiology and pathology of African swine fever. *J. Comp. Pathol.*, **152**, 9–21.

175 TEKLUE T., WANG T., LUO Y., HU R., SUN Y.& QIU H.J. (2020). Generation and Evaluation of an African
176 Swine Fever Virus Mutant with Deletion of the CD2v and UK Genes. *Vaccines*, **8**,
177 763.https://doi:10.3390/vaccines8040763.

178 TIGNON M., GALLARDO C., ISCARO C., HUTET E., VAN DER STEDE Y., KOLBASOV D., DE MIA G.M., LE POTIER
179 M.F., BISHOP R.P., ARIAS M. & KOENEN F. (2011). Development and inter-laboratory validation study of
180 an improved new real-time PCR assay with internal control for detection and laboratory diagnosis of
181 African swine fever virus. *J. Virol. Methods*, **178**, 161–167.

WANG N., ZHAO D., WANG J., ZHANG Y., WANG M., GAO Y., LI F., WANG J., BU Z., RAO Z. & WANG X. (2019). Architecture of African swine fever virus and implications for viral assembly. *Science*, **366**, 640–644. doi: 10.1126/science.aaz1439. Epub 2019 Oct 17

~~ZHANG Y., KE J., ZHANG J., YANG J., YUE H., ZHOU X., QI Y., ZHU R., MIAO F. & LI Q. (2021). African Swine Fever Virus Bearing an I226R Gene Deletion Elicits Robust Immunity in Pigs to African Swine Fever. *J. Virol.*, **95**, e0119921. https://doi: 10.1128/JVI.01199-21.~~

ZHAO D., SUN E., HUANG L., DING L., ZHU Y., ZHANG J., SHEN D., ZHANG X., ZHANG Z., REN T., WANG W., LI F., HE X. & BU Z. (2023). Highly lethal genotype I and II recombinant African swine fever viruses detected in pigs. *Nat. Commun.*, **14**, 3096.

*

* *

NB: There are WOAH Reference Laboratories for African swine fever (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 and reagents for African swine fever

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2021.