ADVICE FOR MEMBER COMMENTS

Chapter Number and Title: 1.1.4/5. Principles and methods of validation of diagnostic assays for infectious diseases

Country making the comments:

Date

NB: The Biological Standards Commission is particularly interested in OIE Member Country opinion on this chapter and its seven appendices: are the documents clear and useful? If not, how they could be improved?

It would be appreciated if the following guidance notes are followed in making a reply:

1. *Comments may be general or specific, but specific comments are usually more valuable. General comments should be such that some conclusion and action can be taken in response to them. For example, it is of little help to make statements such as "This test is no longer used in our laboratory", without indicating the reasons and what test is used instead.*

2. *Specific comments should be identified, by indicating the line number in the text, to facilitate the making of editorial changes.*

3. *The pointing out of typing or technical errors is welcome, but the correct word or figure should be indicated. For example, it is of little help to say that 0.8 M is too high, if the preferred value is not indicated.*

4. *Bear in mind that the introductory chapters are not intended to be exhaustive, and indeed none of the chapters can give a completely comprehensive cover of the subject, otherwise the* Terrestrial Manual *would be too long. However, assistance in indicating priorities is always helpful.*

5. *The* Terrestrial Manual *is intended for world-wide use. The chapters need to reflect the development of new technology, but the old established methods, usually requiring less sophisticated apparatus, have to be retained. New technology should not be described in detail until it has gained wide acceptance as a reliable method.*

General Comments

Specific Comments (add continuation sheets if required)

line:

CHAPTER 1.1.4/5.

PRINCIPLES AND METHODS OF VALIDATION OF DIAGNOSTIC ASSAYS FOR INFECTIOUS DISEASES

INTRODUCTION

Validation is a process that determines the fitness of an assay, which has been properly developed, optimised and standardised, for an intended purpose. Validation includes estimates of the analytical and diagnostic performance characteristics of a test. In the context of this chapter, an assay that has completed the first three stages of the validation pathway (*see Figure 1 below*), *including performance characterisation, can be designated as "validated for the original intended purpose*(s)*"* [1]*. To maintain a validated assay status, however, it is necessary to carefully monitor the assay's daily performance, often by tracking the behaviour of internal controls over time. This ensures that the assay, as originally validated, consistently maintains its performance characteristics. Should it no longer produce results consistent with the original validation data, the assay may be rendered unfit for its intended purpose. Thus, a validated assay is continuously assessed to assure it maintains its fitness for purpose through assessment of results of internal controls in each run of the assay.*

Assay, test method, and test are synonymous terms for purposes of this chapter, and therefore are used interchangeably.

The terms "**valid**" (adjective) or "**validity**" (noun) refer to whether estimates of test performance characteristics are unbiased with respect to the true parameter values. These terms are applicable regardless of whether the measurement is quantitative or qualitative.

Assays applied to individuals or populations have many purposes, such as aiding in: documenting freedom from disease in a country or region, preventing spread of disease through trade, eradicating an infection from a region or country, confirming diagnosis of clinical cases, estimating infection prevalence to facilitate risk analysis, identifying infected animals toward implementation of control measures, and classifying animals for herd health or immune status post-vaccination. A single assay may be validated for one or several intended purposes by optimising its performance characteristics for each purpose, e.g. setting diagnostic sensitivity (*DSe*) *high, with associated lower diagnostic specificity* (*DSp*) *for a screening assay, or conversely, setting DSp high with associated lower DSe for a confirmatory assay.*

The ever-changing repertoire of new and unique diagnostic reagents coupled with many novel assay platforms and protocols has precipitated discussions about how to properly validate these assays. It is no longer sufficient to offer simple examples from serological assays, such as the enzyme-linked immunosorbent assay, to guide assay developers in validating the more complex assays, such as nucleic acid detection tests. In order to bring coherence to the validation process for all types of assays, this chapter focuses on the criteria that must be fulfilled during assay development and validation of all assay types. The inclusion of assay development as part of the assay validation process may seem counterintuitive, but in reality, three of the validation criteria that must be assessed in order to achieve a validated assay, comprise steps in the assay development process. Accordingly the assay development process seamlessly segues into an assay validation pathway, both of which contain validation criteria that must be fulfilled. This chapter also provides guidance on the evaluation of each criterion through provision of best scientific practices contained in the chapter's appendices. The best practices are tailored for each of several fundamentally

1 **Validation** does not necessarily imply that test performance meets any minimum value or that the test has comparable performance to any comparative test, unless this has been specifically considered in the design of the test evaluation study.

different types of assays (*e.g. detection of nucleic acids, antibodies, or antigens*) *and provide more information on specific issues related to the validation of diagnostic assays.*

DIRECT AND INDIRECT METHODS THAT REQUIRE VALIDATION

The diagnosis of infectious diseases is performed by direct and/or indirect detection of infectious agents. By direct methods, the particles of the agents and/or their components, such as nucleic acids, structural or non-structural proteins, enzymes, etc., are detected. The indirect methods demonstrate antibodies or cell-mediated immune responses induced by exposure to infectious agents or their components.

The most common direct detection methods are isolation or *in-vitro* cultivation of viable organisms, electron microscopy, immunofluorescence, immunohistochemistry, antigen-ELISA, Western immunoblotting, and nucleic acid detection systems (NAD). The NAD systems include nucleic-acid hybridisation (NAH), macro- and microarrays and the various techniques of nucleic acid amplification, such as the polymerase chain reaction (PCR), or the isothermal amplification methods, such as nucleic acid sequence-based amplification (NASBA), and invader or loop-mediated isothermal amplification (LAMP). NAD assays are rapidly becoming commonplace and in many cases replacing virus isolation and bacteria cultivation, particularly for the detection of agents that are difficult or impossible to culture. NAD tools are also used as a secondary means for highly specific identification of strains, groups, or lineages of organisms following isolation or culture of viruses, bacteria and parasites. Molecular diagnostics, such as PCR, do not require: a) the presence of replicating organisms, b) expensive viral isolation infrastructure, c) up to several weeks to achieve a diagnosis, or d) special expertise, which is often unavailable in many laboratories – all practical advantages. These methods have become relatively inexpensive, safe and user-friendly (Ballagi-Pordány & Belák, 1996; Belák & Thorén, 2001; Belák, 2005, 2007; Burns *et al*., 2005; Bustin, 2005; Huggett *et al.,* 2005; Lauerman, 2004; Louie *et al.,* 2000). Various real-time PCR methods, nucleic acid extraction robots, and automated workstations for NAD, antibody, antigen, and agent detection have resulted in a large repetoire of high throughput, robust, very rapid and reliable assays. Although NAD systems often have the advantage of a greater diagnostic sensitivity and analytical specificity than infectious agent recovery or antigen-capture ELISA procedures, that advantage usually carries with it a greater challenge for validation of such assays.

The most common indirect methods of infectious agent detection are antibody assays such as classical virus neutralisation, antibody enzyme-linked immunosorbent assay (ELISA), haemagglutination inhibition, complement fixation, and the recently appearing novel methods, such as biosensors, bioluminometry, fluorescence polarisation, and chemoluminescence.

PRELIMINARY CONSIDERATIONS IN ASSAY DEVELOPMENT AND VALIDATION

The first consideration is to define the purpose of the assay, because this guides all subsequent steps in the validation process. By considering the variables that affect an assay's performance, the criteria that must be addressed in assay validation become clearer. The variables can be grouped into three categories: (a) the sample – individual or pooled, matrix composition, and host/organism interactions affecting the target analyte quantitatively or qualitatively; (b) the assay system – physical, chemical, biological and operator-related factors affecting the capacity of the assay to detect a specific analyte in the sample; and (c) the test result interpretation – the capacity of a test result, derived from the assay system, to predict accurately the status of the individual or population relative to the analyte in question.

Assay validation criterion: a characterising trait of an assay; a decisive factor, measure or standard upon which a judgment or decision may be based.

The matrix in which the targeted analyte may reside (serum, faeces, tissue, etc.) may contain endogenous or exogenous inhibitors that prevent enzyme-dependent tests such as PCRs or ELISAs from working. Other factors that affect the concentration and composition of analyte (mainly antibody) in the sample may be mainly attributable to the host and are either inherent (e.g. age, sex, breed, nutritional status, pregnancy, immunological responsiveness) or acquired (e.g. passively acquired antibody, active immunity elicited by vaccination or infection). Non-host factors, such as contamination or deterioration of the sample, also affect the ability of the assay to detect the specific targeted analyte in the sample.

Factors that interfere with the analytical performance of the assay system include instrumentation, operator error, reagent choice (both chemical and biological) and calibration, accuracy and acceptance limits of assay controls, reaction vessels and platforms, water quality, pH and ionicity of buffers and diluents, incubation temperatures and durations, and error introduced by detection of closely related analytes. It is also important that biological reagents are free of extraneous agents.

Factors that may negatively impact diagnostic performance of the assay are primarily associated with choice of reference sample panels from known infected/exposed or known uninfected animals selected for evaluating the

diagnostic sensitivity (DSe) and diagnostic specificity (DSp) of the assay. This is particularly difficult because the degree to which the reference animals represent all of the host and environmental variables in the population targeted by the assay has a major impact on the confidence of test-result interpretation. For example, experienced serologists are aware that an assay, validated by using serum samples from northern European cattle, may not give valid results for the distinctive populations of cattle in Africa. Diagnostic performance of the assay is further complicated when sample panels of known infection status are not available, often because they are impossible to obtain. In this situation, DSe and DSp may be estimated, in certain circumstances by use of latent class models (Enoe *et al*., 2000; Greiner & Gardner, 2000; and Appendix 1.1.4.5).

THE CRITERIA OF ASSAY DEVELOPMENT AND VALIDATION

Assay performance is affected by many factors that span from the earliest stages of assay development through the final stage of performance assessment when the test is applied to targeted populations of animals. An assay, therefore, cannot be considered validated unless a specific set of essential validation criteria (see accompanying box) have been tested and affirmed or fulfilled, either quantitatively or qualitatively (for detail on terms, see the glossary at the beginning of this *Terrestrial Manual*). Lack of attention to any one of these criteria will likely reduce the level of confidence that an assay is fulfilling the purpose(s) for which it is intended. The first four of these criteria typically are addressed during development of the assay (the Development Pathway), and the remaining eight are evaluated during the first three stages of assay validation (the Validation Pathway) as described below.

Assay validation criteria

1. Fitness for intended purpose(s)
2. Optimisation
3. Standardisation
4. Robustness
5. Repeatability
6. Analytical sensitivity
7. Analytical specificity
8. Thresholds (cut-offs)
9. Diagnostic sensitivity
10. Diagnostic specificity
11. Reproducibility
12. Ruggedness

A. ASSAY DEVELOPMENT PATHWAY

1. Definition of the intended purpose(s) for an assay

The OIE *Standard for Management and Technical Requirements for Laboratories Conducting Tests for Infectious Diseases* ~~(25)~~ states that test methods and related procedures must be appropriate for specific diagnostic applications in order for the test results to be of any relevance. In other words, the assay must be 'fit for purpose'[2]. The capacity of a positive or negative test result to predict accurately the infection or exposure status of the animal or population of animals is the ultimate consideration of assay validation. This capacity is dependent on development of a carefully optimised (Section A.2.d), and standardised (Section A.2.g) assay that, through accrual of validation data, provides less biased and more precise estimates of DSe and DSp. These estimates, along with evidence-based data on prevalence of infection in the population being tested, are the basis for providing a high degree of confidence in the predictive values of positive and negative test results. In order to insure that test results provide useful diagnostic inferences about animal population infection/exposure status, the validation process encompasses initial development and assay performance documentation, as well as on-going assessment of quality control and quality assurance programmes. Figure 1 shows the assay validation process, from assay design through the development and validation pathways to implementation, deployment, and maintenance of the assay

~~As outlined in the background information in *Certification of diagnostic assays* on the OIE website (www.oie.int),~~ The first step is selection of an assay type that likely can be validated for a particular use.

a) Fitness for purpose

The most common purposes are to:

1) Demonstrate freedom from infection in a defined population (country/zone/compartment/herd) (prevalence apparently zero):

   1a) 'Free' with and/or without vaccination,

   1b) Re-establishment of freedom after outbreaks

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

2 This is a specific interpretation of the more generally stated requirements of the ISO/IEC 17025:2005 international quality standard for testing laboratories (2005). The OIE Standard further states that in order for a test method to be considered appropriate, it must be properly validated and that this validation must respect the principles outlined in the validation chapters of the *Terrestrial & Aquatic Manuals.*

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

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

5) Estimate prevalence of infection or exposure to facilitate risk analysis (surveys, herd health status, disease control measures).

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

These purposes are broadly inclusive of many narrower and more specific applications of assays (see Appendices for each assay type for details). Such specific applications and their unique purposes need to be clearly defined within the context of a fully validated assay.

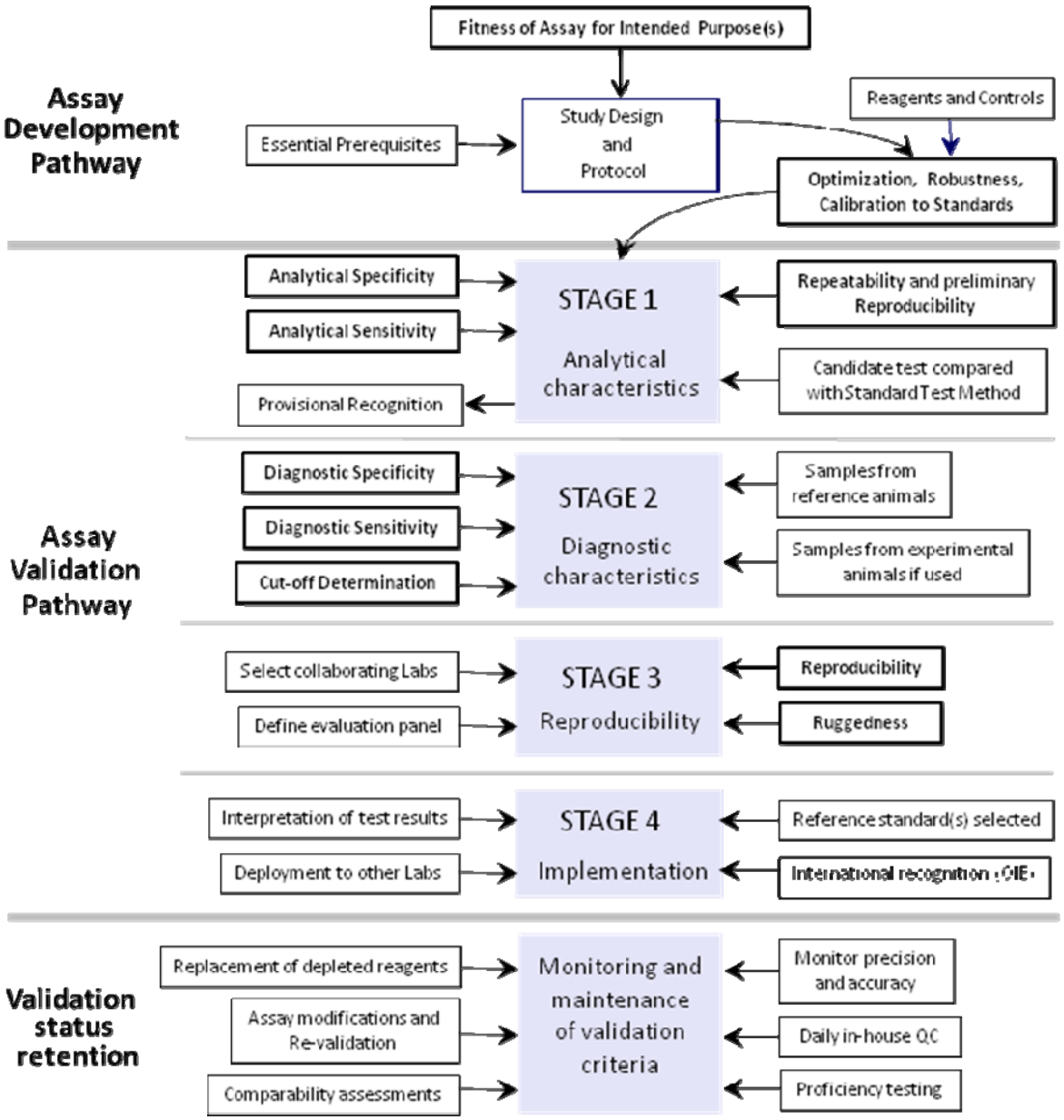

Figure 1. *The assay development and validation pathways with assay validation criteria highlighted in bold typescript within shadowed boxes.*

b) Fitness for use

While this chapter deals with validation and fitness for purpose from a scientific perspective, it should also be noted that other practical factors might impact the relevance of an assay with respect to its intended application. These factors include not only the diagnostic suitability of the assay, but also its acceptability by scientific and regulatory communities, acceptability to the client, and feasibility given available laboratory resources. An inability to meet operational requirements of an assay also may make it unfit for its intended use. Such requirements may include performance costs, equipment availability, level of technical sophistication and interpretation skills, kit/reagent availability, shelf life, transport requirements, safety, biosecurity, sample throughput, turn-around times for test results, aspects of quality control and quality assurance, and whether the assay can practically be deployed to other laboratories. Test kits used in the field are highly desirable from an ease-of-use viewpoint, but because they are performed outside the confines of a controlled laboratory environment, they require added precautions to maintain fitness for purpose (Crowther *et al*., 2006).

2. Assay development – the experimental studies

a) Essential prerequisites: factors that impact assay validation

i) Quality assurance

Whether developing assays in the laboratory or performing analyses of clinical material, the objective is to produce data of high quality. This requires that key requirements have to be fulfilled within the laboratory (see Chapters 1.1.3 & 1.1.1 of the *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* [*Terrestrial Manual*] and the *Manual of Diagnostic Tests for Aquatic Animals,* respectively). The establishment of quality assurance (QA) and quality control (QC) systems is essential, i.e. a set of quality protocols, including the use of assay control samples that ensure that the system is working properly and confirms data reproducibility and quality. QA and QC systems, together with trained and competent personnel, have already been established in many laboratories world-wide.

ii) Equipment selection

Equipment that is not maintained and calibrated can be a major impediment to achieving a quality assay. Apparatus (freezers, heating blocks, incubators, refrigerators, optical colorimeters, thermocyclers, plate washers, pipettes, etc.) must be calibrated according to the laboratory's quality assurance protocols. Examples of this need include robotics used for automation of entire assays, or parts thereof, for routine diagnostic processing. It is not sufficient to assume that robotic extraction of nucleic acid, for example, is equivalent to previously used manual extraction methods or that an automated ELISA plate washer provides uniform washing among wells of the plate. The instrument must be calibrated and the protocol validated to confirm performance efficiency and to assure cross-contamination does not occur in NAD systems or washing is adequate for all wells in a plate. (See Appendices on best practices for more details).

iii) Selection and integrity of samples

Selection, collection, preparation and management of samples are critical variables in designing, developing, and validating an assay. Other variables such as transport, chain of custody, tracking of samples, and the laboratory information management system are also critical sources of variation/error that become especially important when the assay is implemented for routine testing. Integrity of experimental outcomes during assay development and validation is only as good as the quality of the samples used in experimentation or routine diagnostic testing. Anticipating the factors that can negatively impact sample quality must precede launching an assay validation effort. Reference samples used in assay development and validation should be in the same matrix used in the assay (e.g. serum, tissue, whole blood) and representative of the species to be tested by the resulting assay. The reference materials should appropriately represent the range of analyte concentration to be detected by the assay. Details on proper sample collection, preparation, management, and transport are available in the Chapter 1.1.1 of the *Terrestrial Manual*.

b) Test method design and proof of concept

Considerable thought and planning needs to go into designing all steps of a new assay destined for validation, or an existing assay that is being modified. Assistance is offered in Appendices to this chapter, which cover best practices for development and validation of assays for detection of various analytes (e.g. antibody, antigen, and nucleic acid detection).

~~i) Analyte reference samples~~

Development of all assays is dependent on analyte reference samples that reflect the target analyte and the matrix in which the analyte is found in the population for which the assay is intended. The reference samples may be sera, fluids or tissues that contain the analyte of interest or a genomic construct consistent with the

target analyte. These reference materials are used in experiments conducted throughout the development process and carried over into the validation of the assay.

c) Operating range of the assay

Operating range of an assay: an interval of analyte concentrations (amounts) over which the method provides suitable accuracy and precision.

During development of the assay, the lower and upper detection limits are established. To formally establish this range, a high positive reference sample is selected (ideally, this sample will be the same one from among the three samples described under "Optimisation" below). This high positive sample is serially diluted to extinction in an analyte-negative matrix of the same constitution as the sample matrix of samples from animals in the population targeted by the assay. The results are plotted as a 'response-curve', with the response (e.g. OD, Ct, etc.) a function of analyte concentration (amount). The curve, establishes the range of the assay, which is the interval between the upper and lower concentration (amounts) of analyte in the sample for which a suitable level of precision[3] and accuracy has been demonstrated. For most diagnostic assays, the response is the result of interaction of the analyte with an antibody or other binding reagent. These are known as ligand binding assays (LBAs). The typical calibration curve for LBAs is sigmoidal in shape, with a lower boundary (asymptote) near the background response (non-specific binding) and an upper asymptote near the maximum response. Typically, LBA data are transformed to approximate a linear relationship between response and concentration. This transformation simplifies interpolation of data using linear regression analysis, but with the disadvantage of introduced bias. As linearisation is imperfect leading to compromised estimates of accuracy and precision, numerous data-fitting algorithms have been applied to experimental calibration curve data from LBAs (Findlay & Dillard, 2007). The currently accepted reference model for calibration of LBAs is the 4-parameter logistic model, which usually optimises accuracy and precision over the maximum usable calibration range (Findlay & Dillard, 2007). Such transformations are now more practical for the general user because of many user-friendly statistical software programs available on the internet.

Precision is the degree of dispersion among a series of measurements of the same sample tested under specified conditions (see footnote 3 for more detail).

Accuracy is the closeness of a test value to the expected (true) value for a reference standard reagent of known concentration or titre.

d) Optimisation

It is useful to select at least three well-defined reference samples, representing the analyte ranging from high positive to negative (e.g. negative, low- and high-positive). These samples ideally should represent known infected and uninfected animals from the population that eventually will become the target of the assay once it is validated. Obtaining such reference samples, however, is not always possible, particularly for nucleic acid and antigen detection assays. The alternative of preparing reference samples spiked with cultured agents or positive sera is inferior because the matrix of field samples may be very different from spiked-sample matrix. But, when no other alternative exists, spiking a sample with a known amount of the analyte derived from culture, or diluting a high positive serum in negative serum of the same species may be all that is available. In either case, it is imperative that the matrix, into which analyte is placed or diluted, is identical to, or resembles as closely as possible the samples that ultimately will be tested in the assay. Ideally, reference samples have been well characterised by one or preferably at least two alternate methodologies. These samples can be used in experiments to determine if the assay is able to distinguish between varying quantities of analyte, and for optimising the reagent concentrations and perfecting the protocol. In principle, for all assay types, it is highly desirable to prepare and store a sufficient amount of each reference sample in aliquots for use in every run of the candidate assay as it is evaluated through the entire development and validation process. 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.

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.

The labour-intensive process of optimising an assay is fundamental and critical to achieving a quality assay. Scientific judgment and use of best scientific practices provided in accompanying Appendices to this chapter are the best assets to guide optimisation of all elements of an assay. The approach outlined provides a solid foundation for development of an assay that fulfils the criteria of "robustness" and "ruggedness" when used

3 Precision may be evaluated in several ways by testing the same replicated sample: 1) within a plate or plates in a run of the assay, 2) between plates run concurrently within a run of the assay, 3a) between assay runs at different times in the same day or on different days under similar conditions, 3b) between assay runs on different days with different operators, 4) between laboratories. In this chapter, precision categories 1–3 are estimates of repeatability, and precision category 4 is synonymous with reproducibility. Levels 3a and 3b are also known as intermediate precision.

over extended periods of time within a laboratory or when implemented in other laboratories. Often, prototype assays are developed using reagents and equipment at hand in the laboratory. However, if the assay is intended for routine diagnostic use in multiple laboratories, optimisation becomes extremely critical. Every chemical and buffer formulation must be fully described. All reagents must be defined with respect to purity and grade (including water). Acceptable working ranges must be established for parameters such as pH, molarity, etc. Likewise for biologicals, standards for quality, purity, concentration and reactivity must be defined. Shelf lives and storage conditions must also be considered for both chemicals and biologicals. Acceptable ranges for reaction times and temperatures also need to be established. Essential equipment critical to assay performance must be described in detail, including operational specifications and calibration. Process (quality) control is often an add-on at the end of assay development, but it should be an integral part of optimisation from the very beginning. In addition to the above, downstream aspects such as data capture, manipulation and interpretation may also require standardisation and optimisation. Finally, all of these parameters, once optimised, must be fully described in the test method protocol.

In some assay types, a correct assay result is fully dependent on getting a particular step in the testing process correct, requiring special attention during optimisation. A case in point is nucleic acid extraction from the sample. Both commercial (robotic, spin columns, and magnet-based extractions, etc.) and standard chemistry-based methods are used for DNA or RNA extraction. It is crucial to determine the most reproducible and efficient extraction method through optimisation experiments. Extraction needs to be optimised for every type of tissue that may be targeted by the assay. If the method of extraction is changed, at a minimum, comparable efficiency of extraction should be demonstrated (see Section B.6 below and associated Appendix 1.1.4.6 for additional information on establishing comparability when reagents are changed).

A variety of analyte reference samples and other process controls that are routinely included in any assay system are identified in the following sections. These provide critical assay monitoring functions that require special attention during assay optimisation. In addition, attention must be paid to the proper preparation and storage of all biological reagents and reference materials to ensure stability (see Chapter 1.1.1 of this *Terrestrial Manual*).

During experimentation to optimise the assay, take note of assay parameters that have a narrow range in which they perform optimally, as these are the critical points that may affect an assay's robustness (see Section A.2.f).

e) Inhibitory factors in sample matrix

Generally, for antibody detection in serum, assays are rather resistant to inhibitory factors, with the exception of certain assays, e.g. toxic factors in viral neutralisation assays, or when endogenous substances found in certain sample types inhibit enzymatic reactions in ELISAs. For nucleic acid detection, sample matrices including blood, serum, body tissues, and swab samples allow for easy extraction of target nucleic acids, while faeces, autolysed tissues and semen samples can be more difficult to handle because of the presence of factors that can inhibit downstream assays such as PCR.

f) Robustness

Robustness is a measure of an assay's capacity to remain unaffected by small, but deliberate, variations in method parameters, and provides an indication of its reliability during normal usage.

Robustness refers to an assay's capacity to remain unaffected by minor variations in test situations that may occur over the course of testing in a single laboratory. It is assessed by deliberate variations in method parameters (International Conference on Harmonisation [ICH], 2005). Assessment of robustness should begin during assay development and optimisation stages. The deliberate variations in method parameters may be addressed in experiments after optimal conditions for an assay are established. However, when multi-factorial titrations of reagents are used for optimising the assay, indications of a compromised robustness may surface. If slight differences in conditions or reagent concentrations cause unacceptable variability, the assay most likely will not be robust. Early knowledge of this situation elicits a critical decision point for determining whether to continue with validation of the assay would be worthwhile, because if an assay is not robust within one laboratory under rather ideal conditions, it is unlikely to exhibit ruggedness (reduced reproducibility) when transferred to other laboratories (ruggedness is addressed in Section 4).

The factors most likely to affect assay robustness are quantitative (continuous) such as pH and temperature; qualitative (categorical) such as batch of reagents or brand of microtitre plates; and mixture-related such as aqueous or organic matrix factors (Dejaegher & Vander Heyden, 2006). For ligand-binding assays (LBAs), lack of robustness is not only due to less-than-optimal concentration/amount of the bio-reagent specified in the method, but may also be due to the intrinsic characteristics of the biological reagent (e.g. monoclonal antibody affinity or polyclonal antibody avidity and/or valency). Robustness, therefore, particularly of LBA-based assays, may be affected by systematic and/or random errors (Thompson *et al.,* 2002).

Robustness testing is demonstrated on a method-by-method basis. All critical reagents are identified and subjected to a factorial ~~assessment~~ design experiments, which compares all possible combinations of reagents (Dejaegher & Vander Heyden, 2006; Youden & Steiner, 1987). For example, in antibody detection by ELISA, factors may include concentration of antigen bound to the solid phase, conjugate dilution and several test sera representing the operating range of assay. The response of the assay with respect to these small changes shall not result in unacceptable variability. ~~Alternatively, robustness can be demonstrated through the application of factorial design experiments (9, 26).~~

Robustness is further verified during Stage 1 of assay validation. When the optimised test is first run under routine laboratory conditions, this practical measure of robustness is referred to as repeatability (see Section 2.a) and it is continually monitored as part of process control procedures for the duration of the life of the assay (see Section 6.a).

g) Calibration of the assay to standard reagents

i) International and national analyte reference standards

Ideally, international reference standards, containing a known concentration of analyte, are the reagents to which all assays are standardised. Such standards are prepared and distributed by international reference laboratories. National reference standards are calibrated by comparison with an international standard reagent whenever possible; they are prepared and distributed by a national reference laboratory. In the absence of an international reference standard, a national reference standard becomes the standard of comparison for the candidate assay. These standard reagents are highly characterised through extensive analysis, and preferably the methods for their characterisation, preparation, and storage have been published in peer-reviewed publications.

ii) In-house standard reagent

An in-house reference standard generally has the highest metrological quality available at a given location in a given organisation, and is calibrated against an international or national standard. In the absence of either of these calibrators and to the extent possible, the in-house standard is highly characterised in the same manner as international and national analyte standards. This local in-house standard therefore becomes the best available standard, and is retained in sufficient aliquotted volumes for periodic use as the standard to which working standards are calibrated.

iii) Working standard reagent

One or more working standard reagent(s), commonly known as analyte or process controls, are calibrated to an international, national, or in-house standard reagent, and are prepared in large quantities, aliquotted and stored for routine use in each diagnostic run of the assay.

h) "Normalising" test results to a working standard(s)

Due to the inherent variation in raw test results that are often observed between test runs of the same assay or among laboratories using the same or similar assays, it is virtually impossible to directly compare (semi-) quantitative data. To markedly improve the comparability of test results both within and between laboratories, one or more working standard reagent(s) are included in each run of an assay. Raw test values for each test sample can then be converted to units of activity relative to the working standard(s) by a process called 'normalisation' [not to be confused with transformation of data to achieve a 'normal' (Gaussian) distribution]. The 'normalised' values may be expressed in many ways, such as a per cent of a positive control (e.g. in an ELISA), or as a concentration or titre of an analyte derived from a standard curve, or as a number of targeted genomic copies also derived from a standard curve of Ct (cycle threshold) values for real time PCR. It is good practice to include working standards [or at least a reasonably well-characterised sample(s) in all runs of the assay during assay development and validation because this allows 'normalisation' of data, which provides a valid means for direct comparison of results between runs of an assay. Automated assay systems may calculate and report 'normalised' data by, for example, a standard curve, or by reporting the cycle number at which the cycle threshold is exceeded as in real-time PCR. For more information, see Appendices 1.1.4.1 and 1.1.4.3.

B. ASSAY VALIDATION PATHWAY

1. Definition of a validated assay

"Validation" is a process that determines the fitness of an assay that has been properly developed, optimised and standardised for an intended purpose. Validation includes estimates of the analytical and diagnostic performance characteristics of a test. In the context of this document, an assay that has completed the first three stages of the validation pathway (Figure 1), including performance characterisation, can be designated as "validated for the original intended purpose(s)"

To retain the status of a validated assay, however, it is necessary to assure that the assay as originally validated consistently maintains the performance characteristics as defined during validation of the assay (see Section 6 below). This can be determined in a quality assurance program characterised by carefully monitoring the assay's daily performance, primarily through precision and accuracy estimates for internal controls, and by scheduled external proficiency testing. Should the assay cease to produce results consistent with the original validation data, the assay would be rendered unfit for its intended purpose. Thus, a validated assay must be continuously assessed to assure it maintains its fitness for purpose.

2. Stage 1 – Analytical performance characteristics

Ideally, the design for studies outlined in the following sections should be done with assistance of a statistician. It is possible to design experiments that efficiently provide information on likely within- and among-laboratory sources of variation in assay precision (see footnote 3 under Section A.2.c, above), which will define the performance characteristics of the assay. Stage 1 studies for repeatability, reproducibility and assessment of analytical sensitivity (limit of detection) should be performed in a blinded fashion with random selection of samples. The choice of organisms, strains or serotypes to assess analytical specificity should reflect current knowledge and therefore inform the best possible experimental design for targeting specific analytes.

a) Repeatability

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. Each of these samples is then aliquoted into individual vessels as three identical replicates of the original sample containing the original analyte and matrix concentration. Each replicate is then run through all steps of the assay, including creating the working dilution, as though it were a test sample derived from the population targeted by the assay. It is not acceptable to prepare a final working dilution of a sample in a single tube from which diluted aliquots are pipetted into reaction vessels, or to create replicates from one extraction of nucleic acid rather than to extract each replicate before dilution into the reaction vessels. Such 'samples' do not constitute valid replicates for repeatability studies. Between-run variation is determined by using the same samples in multiple runs (approximately 20) involving two or more operators, done on at least five separate days. The variation in replicate results can be expressed as standard deviations, confidence intervals, or other possible options (see Appendix 1.1.4.4 on ~~measures of~~ Measurement uncertainty for assessments of repeatability).

b) Analytical specificity (ASp)

Analytical specificity is the degree to which the assay distinguishes between the target analyte and other components that may be detected in the assay.

Analytical specificity distinguishes between the target analyte and other components in the assay in at least three distinctive ways. These are described as the selectivity, exclusivity, and inclusivity of the assay.

- **Selectivity** refers to the extent to which a method can accurately quantify the targeted analyte in the presence of: 1) interferents such as matrix components (e.g. inhibitors of enzymes in the reaction mix); 2) degradants (e.g. toxic factors); 3) non-specific binding of reactants to a solid phase (e.g. conjugate of an ELISA adsorbed to well of microtiter plate); 4) antibodies to vaccination that may be confused with antibodies to active infection. Such interferents may cause falsely reduced or elevated responses in the assay that negatively affect its analytical specificity. Vessman *et al.* (2001) is a useful overview of selectivity as defined for analytical chemistry from which a modification described herein was deduced for application to diagnostic tests.

- **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 would also define a confirmatory assay.

- **Inclusivity** is the capacity of an assay to detect several strains or serovars of a species, several species of a genus, or a similar grouping of closely related organisms or antibodies thereto. It characterises the scope of action for a screening assay.

After eliminating interferents to the extent possible, the next step is to test a panel of samples appropriate for evaluating either inclusivity, exclusivity, or both, depending on the intended purpose of the assay.

ASp applies to both direct and indirect methods of analyte detection. If exclusivity is required, field samples should be obtained from animals infected with genetically-related, non-pathogenic organisms, but this may prove difficult or even impossible. In such cases, cultivated organisms can be used in direct detection methods, or serum from animals exposed experimentally by natural routes for indirect detection methods.

Acceptable cross-reactivity is largely dependent on the intended purpose of the test and the prevalence of the cross-reactive organisms/analytes in the target population samples – which must be determined for each case (see Appendix 1.1.4.5 for more detail). For PCR, it is useful to perform computer simulation studies as an adjunct to laboratory assessment of ASp; however, such studies are not sufficient by themselves to evaluate ASp.

A factor unique to viral antibody detection is the possible antibody response of animals to carrier proteins found in vaccines – another type of interferent that may negatively affect selectivity. If such proteins are also present in the solid phase antigen on ELISA plates, they may bind antibody developed to vaccine carrier proteins and give false-positive results (lack of exclusivity in the assay). Use of vaccine preparations as antigens in ELISAs is, therefore, not recommended. See Appendix 1.1.4.1 for specific practices to determine ASp.

c) Analytical sensitivity (ASe)

Analytical sensitivity is synonymous with the lower limit of detection (LOD) of an analyte in an assay. For direct-detection assays, this may be expressed as the number of genome copies, infectious dose, colony-forming units, plaque forming units, etc., of the agent that can be detected and distinguished from the result of a matrix background. Most commonly, this is expressed as a number of copies, complement-fixing units or plaque-forming units giving at least 50% positive results among the replicates of a sample for a specified volume or weight (see Appendix 1.1.4.5 for detail on LOD determination). For indirect detection assays, it is the least amount of antibody detected, usually, the penultimate dilution of sample in which the analyte is indistinguishable from the activity of a sample matrix control.

Limit of detection or analytical sensitivity, is the smallest amount of analyte in a sample that can be detected by a direct detection assay in at least 50% of the replicates for each dilution, in a dilution series of analyte in matrix.

If the intended purpose is to detect low levels of analyte or subclinical infections and it is difficult to obtain the appropriate reference materials, for example samples from early stages of the infection process, it may be useful to determine comparative analytical sensitivity by running a panel of samples on the candidate assay and on another independent assay. This would provide a relative comparison of analytical sensitivity between the assays, but care must be taken in choosing the independent assay used in the 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.

Where a new, more sensitive test is developed, it may be necessary to test serial samples taken from infected animals early after infection, on through to the development clinical or fulminating disease, and to run these in parallel with previously used tests to demonstrate the increased sensitivity. This would also provide a temporal comparison of the earliest point of detection relative to the pathogenesis of the disease.

d) Standard test method for comparison with the candidate assay test method

There are situations where it is not possible or desirable to continue to 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 on the candidate assay method and by the standard method if it exists.

A **Standard test method** is the best internationally or nationally recognised method, or in their absence, the best available method preferably published in a reputable journal.

e) Analytical accuracy of adjunct tests or procedures

Some test methods or procedures may be qualified for use as analytical tools in the diagnostic laboratory. These usually are secondary adjunct tests or procedures that are applied to an analyte that has been detected in a primary assay. The purpose of such analytical tools is to further characterise the analyte detected in the primary assay. Examples of such adjunct tests include virus neutralisation to type an isolated virus, or molecular sequencing to confirm a real-time PCR test result. Pathogenicity indices, haemagglutination inhibition, drug resistance determinations, etc. are other examples where adjunct tests or procedures are performed, either independent of, or as part of a primary assay.

Such adjunct tests must be validated for analytical performance characteristics (Sections A.2 through B.2.c., above), but differ from routine diagnostic tests because they do not require validation for diagnostic performance characteristics (Sections B.3 through B.5, below). The analytical accuracy of these tools may be defined by comparison with a reference reagent standard, or by characteristics inherent in the tool itself (such as endpoint titration). In all of these examples, the targeted analyte is further characterised quantitatively or qualitatively by the analytical tool.

f) Preliminary evaluation of reproducibility

Preliminary reproducibility estimates of the candidate assay may be useful at this time in the validation process, where only a small panel of highly characterised samples is available. This panel could be used for a limited evaluation of reproducibility to enhance provisional acceptance status for the assay. The candidate test method is then duplicated in laboratories at one or more different institutes, and the panel of samples is evaluated using the candidate assay in each of these laboratories, using the same protocol, same reagents as specified in the protocol, and comparable equipment. This is a scaled-down version of Stage 3 of assay validation.

g) Provisional assay recognition[4]

Provisional recognition defines an assay that has been assessed through Stage 1 for critical assay benchmark parameters: ASe, ASp, repeatability, and an estimate of reproducibility.

Experience has shown that the greatest obstacle for continuing through Stage 2 of the Validation Pathway is the number of defined samples required to calculate DSe and DSp (see requirements for Stage 2, Diagnostic Performance, below). The formula is well known and tables are available for determining the number of samples required to estimate various levels of DSe and DSp, depending on the amount of allowable error and the level of confidence in the estimates (Table 1 and Jacobson, 1998). The formula assumes that the myriad of host/organism factors that may affect the test outcome are all accounted for. Since that assumption may be questionable, the estimated sample sizes are at best minimal. For a disease that is not endemic or widespread, it may be impossible, initially, to obtain the number of samples required, but over time, accrual of additional data will allow adjustment of the threshold or if no adjustment is needed, enhance confidence in the estimates.

Historical precedent would suggest that assays were generally the product of laboratory experiments with an emphasis on analytical sensitivity and analytical specificity, and evaluation of panels of field samples was nominal. Such bench validation for bovine spongiform encephalopathy (BSE) is a classical example where positive field samples were not available. Nevertheless, during extended periods of usage in diagnostic settings, such tests have undergone adjustments based on empirical evidence to reduce false-positive and false-negative results. ~~For some of the BSE rapid tests, adjustments had to be made in the cut-off to reduce false-positive results apparent in early implementation. Bench validation provided a level of confidence in diagnostic performance that was adequate for conditional diagnostic use as determined by national authorities.~~ But, this must never become a replacement for a full field validation. Therefore bench validation of diagnostic assays can only offer provisional recognition with anticipation that full field validation will follow.

A provisional recognition of an assay by state or national authorities ~~recognises~~ means that the assay has not been evaluated for diagnostic performance characteristics. As such, the laboratory should develop and follow a protocol for adding and evaluating samples, as they become available, to fulfil this requirement. Ideally, this process should be limited to a specific timeframe in which such an accrual would be directed toward fulfilling Stages 2 and 3 of the validation pathway, and to particular situations (emergencies, minor species, no other test available, etc.). ~~This concept should be limited to emergency situations where rapid introduction of new tests is deemed essential by authorities. There may be other situations where bi-lateral trade agreements, based on tests (e.g. standard test methods) that have not been fully validated within a given country, are mutually accepted. In exceptional cases for rare diseases where no other assay option exists, provisional recognition may be allowed, but reporting of results should include a statement of the provisional nature of the validation of the assay~~. In all cases, sound evidence must exist for preliminary estimates of DSp and DSe based on a small select panel of well-characterised samples containing the targeted analyte.

3. Stage 2 – Diagnostic performance of the assay

Diagnostic Sensitivity and Diagnostic Specificity: Estimates of DSe and DSp are the primary performance indicators established during validation of an assay. These estimates are the basis for calculation of other parameters from which inferences are made about test results (e.g. predictive values of positive and negative test results). Therefore, it is imperative that estimates of DSe and DSp are as accurate as possible. Ideally, they are derived from testing a panel of samples from reference animals, of known history and infection status relative to the disease/infection in question and relevant to the country or region in which the test is to be used. Receiver operating characteristic curve analysis is a useful adjunct to estimation of DSe and DSp because it assesses the global accuracy of a quantitative diagnostic test across possible assay values (Zweig & Campbell, 1993). This approach is described in-depth in Appendix 1.1.4.5.

4 Provisional recognition does not imply certification by the OIE. It does, however, recognise an informed decision of authorities at local, state, national or international levels of their conditional approval of a partially validated assay~~, usually for a time-limited use in emergency situations or as the basis for bi-lateral agreements between countries that choose to accept results from such an assay for trade purposes~~.

Diagnostic sensitivity is the proportion of samples from known infected reference animals that test positive in an assay.

Diagnostic specificity is the proportion of samples from known uninfected reference animals that test negative in an assay.

A sampling design must be chosen that will allow estimation of DSe and DSp. The designated number of known positive and known negative samples will depend on the likely values of DSe and DSp of the candidate assay and the desired confidence level for the estimates (Table 1 and Jacobson, 1998). An abbreviated Table 1 provides two panels of the theoretical number of samples required, when either a 5% or 2% error is allowed in the estimates of DSe or DSp. Comparison of a 5% vs 2% error shows a considerable reduction in the number of samples required. A rather large number of samples are required to achieve a very high confidence for DSe and DSp when a minimal amount of error in the estimate is desired. Logistical and financial limitations may require that less than an optimal number of samples will be evaluated. However, by reducing the DSe and DSp confidence levels to less than 90% usually would not be recommended. Sample size also may limited by the fact that reference populations and "gold standards" (perfect reference standards) may be lacking (see Appendix 1.1.4.5 for further detail). It may, therefore, be necessary to use a sub-optimal number of samples initially. It is, however, highly desirable to enhance confidence and reduce allowable error in the DSe and DSp estimates by adding more samples as they become available.

Table 1. *Theoretical number of samples from animals of known infection status required for establishing diagnostic sensitivity (DSe) and specificity (DSp) estimates with known confidence*

Estimated DSe or DSp	**2%** error allowed in estimate of DSe and DSp						**5%** error allowed in estimate of DSe and DSp					
	Confidence						Confidence					
	75%	80%	85%	90%	95%	99%	75%	80%	85%	90%	95%	99%
90%	257	369	475	610	864	1493	41	59	76	98	138	239
92%	210	302	389	466	707	1221	34	48	62	75	113	195
94%	161	232	298	382	542	935	26	37	48	61	87	150
95%	136	196	251	372	456	788	22	31	40	60	73	126
96%	110	158	203	260	369	637	18	25	32	42	59	102
97%	83	119	154	197	279	483	13	19	25	32	45	77
98%	56	80	103	133	188	325	9	13	16	21	30	52
99%	28	41	52	67	95	164	4	7	8	11	15	26

Per cent error allowed in the estimate of DSe or DSp = 2% in the left panel and 5% in the right panel. For the number of samples required for 1%, 3%, and 4% allowable error in the estimate of DSe and DSp, multiply the number of samples in the left panel of the table by a factor of 4.0, 0.44, and 0.25, respectively.

The following are examples of reference populations and methodologies that may aid in determining performance characteristics of the test being validated.

a) Reference animal populations

Ideally, selection of reference animals requires that important host variables in the target population are represented in animals chosen for being infected with or exposed to the target agent, or that have never been infected or exposed. The variables to be noted include but are not limited to species, age, sex, breed, stage of infection, vaccination history, and relevant herd disease history**.**

i) Negative reference samples

True negative samples, from animals that have had no possible infection or exposure to the agent, may be difficult to locate. It is often possible to obtain these samples from countries that have eradicated or have never had the disease in question. Such samples are useful as long as the targeted population for the assay is similar to the sample-source population.

ii) Positive reference samples

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 another test such as a nucleic acid detection system.

iii) Samples from animals of unknown status

See Appendix 1.1.4.5 Section 5.b.i, and Section 3.b.ii of this chapter for a discussion of latent class models.

b) Reference animal infection status

i) So-called "Gold Standard" model

The term 'gold standard' is commonly used to describe any standard of comparison, but it should be limited to methods or combination of methods that unequivocally classify animals as infected/exposed or uninfected. Some isolation methods themselves have problems of repeatability and analytical sensitivity, so are not truly gold standards particularly for purportedly negative samples. When the so-called reference standard is imperfect, which is the common scenario for most ante-mortem tests, estimates of DSe and DSp for the candidate assay may be compromised because the error in estimates obtained by comparison to the relative standard is carried over into the estimates for the candidate assay. Indeed, when using imperfect reference assays, the DSe and DSp performance estimates of the candidate assay will be flawed and often overestimated.

NAD assays may be more sensitive and specific than existing 'gold standard' methods, which render the established 'gold standard' as not suitable for use as a comparison. If the NAD is more sensitive than the 'gold standard,' an apparent lower relative specificity will be misleading. This problem may be partially resolved by assessing sample derivation, clinical history and sequencing of any PCR products to confirm analyte identity.

ii) Latent-class models

Latent-class models (Branscum *et al*., 2005; Enoe *et al*., 2000; Georgiadis *et al.,* 2003; Hui & Walter, 1980) do not rely on the assumption of a perfect reference test but rather estimate the accuracy of the candidate test and the reference standard with the joint 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 (see Appendix 1.1.4.5 on statistical considerations for details).

c) Experimentally infected or vaccinated reference animals

Sera obtained sequentially from experimentally infected or vaccinated animals are useful for determining the kinetics of antibody responses or the presence/absence of antigen or organisms in samples from such animals. However, multiple serially acquired pre- and post-exposure results from individual animals are not acceptable for establishing estimates of DSe and DSp because the statistical requirement of independent observations is violated. Only single time-point sampling of individual experimental animals is acceptable. Also, for indirect methods of analyte detection, exposure to organisms under experimental conditions, or vaccination may elicit antibody responses that may not be quantitatively and qualitatively typical of natural infection in the target population (Jacobson, 1998). The strain of organism, dose, and route of administration to experimental animals are examples of variables that may introduce error when extrapolating DSe and DSp estimates to the target population. For these reasons, validation of an assay should not be based solely on samples from experimental animals.

d) Threshold (cut-off) determination

To obtain DSe and DSp estimates of the candidate assay, the test results first must be reduced to categorical (positive, negative, or intermediate) status. This is accomplished by insertion of one or two cut-off points (threshold or decision limits) on the continuous scale of test results. The selection of the cutoff(s) should reflect the purpose of the assay and its application, and must support the required DSe and DSp of the assay. Options and descriptive methods for determining the best way to express DSe and DSp are available (Branscum *et al*., 2005; Georgiadis *et al.,* 2003; Greiner *et al.,* 1994; Greiner *et al.,* 2000; Jacobson, 1998; Zweig & Campbell, 1993 and Appendix 1.1.4.5 on statistical considerations). If considerable overlap occurs in the distributions of test values from known infected and uninfected animals, it is difficult to select a single cut-off that will accurately classify these animals according to their infection status. Rather than a single cut-

Threshold and cut-off are considered to be synonymous. A cut-off is the test value selected for distinguishing between negative and positive results on a continuous scale of test values.

Intermediate, inconclusive, suspicious, or equivocal are terms used synonymously for a zone of test values between the positive and negative cut-offs.

off, two cut-offs can be selected that define a high DSe (e.g. inclusion of 99% of the values from infected animals), and a high DSp (e.g. 99% of the values from uninfected animals). The values that fall between these percentiles may be classified as intermediate (see box), and would require testing by a confirmatory assay, retesting for detection of sero-conversion, or sequencing for identity.

The main difficulty in establishing cut-offs based on diagnostic performance characteristics is the lack of availability of the required number of well-characterised samples. Alternatives are discussed in Section B.2.g. on provisional acceptance of an assay during accrual of data to enhance estimates of DSe and DSp.

e) Calculation of DSe and DSp based on test results of reference sera

A typical method for determining DSe and DSp estimates is to test the reference samples in the new assay, and cross tabulate the categorical test results in a 2 × 2 table. In a hypothetical example, assume the test developer decided that estimated DSe and DSp for the new assay should be 97% and 99%, respectively, with a desired confidence of 95% for both estimates. The amount of allowable error in the estimates was set at 2%. Table 1 indicates that 279 samples from known infected animals are required for the DSe assessment, and 95 known negative samples are needed for establishing the DSp estimate. The samples were then run in the new assay. Table 2 is a hypothetical set of results and the calculated DSe and DSp estimates based on the samples tested.

Table 2. *Diagnostic sensitivity and specificity estimates calculated from hypothetical set of results for samples tested from known infected and non-infected populations*

		Number of reference samples required*	
		Known positive (279)	Known negative (95)
Test results	Positive	270 (TP)	7 (FP)
	Negative	9 (FN)	88 (TN)
		Diagnostic sensitivity*	Diagnostic specificity*
		TP/(TP + FN)	TN/(TN + FP)
		96.7% (94.0 – 98.5%)**	**92.0%** (84.3 – 96.7%)**

*Based on Table 1 for an assay with the following parameters:
1) Prior to testing, estimated DSe of 97% and DSp of 99%
2) 95% = required confidence in DSe and DSp estimates
3) 2% = Allowable error in the estimates of DSe and DSp

TP and FP = True Positive & False Positive, respectively
TN and FN = True Negative and False Negative, respectively
** 95% exact binomial confidence limits for DSe and DSp calculated values (see Appendix 1.1.4.5 for information on confidence limits)

In this example, the DSe estimate is as anticipated, but the DSp is much reduced from the anticipated 99%. As a consequence, the width of the confidence interval for DSp is greater than expected. Re-inspection of Table 1 indicates that 707 samples are necessary to achieve an error margin of ± 2% at a DSP of 92% but such an increase in sample size might not be feasible

4. Stage 3 – Reproducibility and augmented repeatability estimates

Ruggedness is a measure of the assay's capacity to remain unaffected by substantial changes or substitutions in test conditions anticipated in multi-laboratory utilisation.

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.

Reproducibility is an important measure of the precision of an assay when used in several laboratories located in distinct or different regions or countries using the identical assay (protocol, reagents and controls). Each of at least three laboratories test the same panel of samples (blinded) containing a minimum of 20 samples, with identical aliquots going to each laboratory (see Appendix 1.1.4.7 on panels of samples). This exercise also generates preliminary data on non-random effects attributable to deployment of the assay to other laboratories - also known as ruggedness of the assay. In addition, within-laboratory repeatability estimates are augmented by the replicates used in the reproducibility studies. Measurements of precision can be estimated for both the reproducibility and repeatability data (see Appendix 1.1.4.4 on Measurement of Uncertainty for further explanation of the topic and its application).

5. Stage 4 – Programme implementation

a) Interpretation of test results

Predictive values of test results: An assay's test results are most useful when the inferences made from them are accurate. Predictive values for test results need to be based on the true prevalence of exposure/infection in the targeted population. For screening assays used in surveillance of a 'disease-free' population, false-positive results are a significant problem. For instance, an assay may have impeccable credentials (e.g. high precision and accuracy, 99% DSe and 99.9% DSp). But if the prevalence of disease is close to zero, and the assay has one false positive for every 1000 animals tested, false-positive inferences are a problem (see Jacobson, 1998 for PV tables for various estimates of DSe and DSp). Similarly, if the assay for a highly virulent disease has one false negative for every 100 animals, false negative inferences could have devastating consequences. This illustrates the critical importance of choosing diagnostic thresholds that are appropriate for the application at hand. Thresholds should be chosen to minimise the effect of false positives and/or false negatives on the predictive values of the test given its application and the prevalence of exposure/infection in the target population. It may also be prudent to have highly specific confirmatory assays to determine whether screening assay reactors are true or false positives.

Predictive Value (PV) of positive or negative test result. PV+ is the probability that an animal has been exposed or infected, given that it tests positive. PV is the probability that an animal is free from exposure or infection, given that it tests negative.

For nucleic acid assays, it may be necessary to confirm NAD-positive results by sequence analysis of the amplified product (an example of an assay to assist in resolving errors due to non-specific target or primer binding).

b) International recognition

Traditionally, assays have been recognised internationally by the OIE when they are designated as prescribed or alternate tests for trade purposes. This has often been based on evidence of their usefulness on a national, regional or international basis. For ~~test~~ commercial diagnostic kits that have ~~completed the certification process~~ gone through the OIE procedure for validation and certification of diagnostic assays, the final step is listing of the test in the OIE Register. Tests listed in the Register are certified as fit for a specific purpose if they have completed Validation Stages 1, 2 and 3. The Register is intended to provide potential ~~test~~ kit users with an informed and unbiased source of information about the ~~test~~ kit and its performance characteristics for an intended purpose. The Register is available on the OIE website at: http://www.oie.int/en/our-scientific-expertise/certification-of-diagnostic-tests/the-register-of-diagnostic-tests/

c) Deployment of the assay

Ultimate evidence of the usefulness of an assay is its successful application(s) in other laboratories and inclusion in national, regional and/or international programmes. Reference laboratories play a critical role in this process. In the natural progression of diagnostic and/or technological improvements, new assays will become the new standard method to which other assays will be compared. As such, they may progressively achieve national, regional and international recognition. As a recognised standard, these assays will also be used to develop reference reagents for quality control, proficiency and harmonisation purposes. These reference reagents may also become international standards.

An assessment of ruggedness should be repeated when the test is transferred from the development laboratory to the field, whether for use in local laboratories or in pen-side applications. Predictable changes, e.g. extremes of temperature and levels of operator experience, should be assessed as additional sources of variation in assay results that may affect estimates of ruggedness (which is mostly derived from reproducibility estimates).

6. Monitoring assay performance after initial validation

a) Monitoring the assay

A validated assay in routine use needs to be consistently monitored for repeatability through process controls to evaluate possible temporal changes in test precision and accuracy. These changes can be monitored graphically by plotting control values in control charts. Deviations from the expected performance should be investigated so corrective action can be taken if necessary. Such monitoring provides critical evidence that the assay retains its "validated" designation during the implementation phase of the assay. Subsequent ongoing evaluation of the assay's performance is also essential and is usually done through assessments of precision, accuracy, and outlier tendencies using control charts. Reproducibility is assessed through external quality control programmes such as proficiency testing.

Control chart: A graphical representation of data from the repetitive measurement of a control sample(s) tested in different runs of the assay over time.

b) ~~Diagnostic~~ Modifications and enhancements – considerations for changes in the assay

Over time, modification of the assay likely will be necessary to address changes in the analytes targeted (i.e. modification of the assay to adjust diagnostic performance) or technical modifications may be needed to improve assay efficiency or cost-effectiveness.

If the assay is to be applied in another geographical region and/or population, revalidation of the assay under the new conditions is recommended. Lineages or sub-lineages of a virus, derived from animals in different geographic locations, are known to have different target sequences or primer sites, requiring revalidation of the assay. This is especially true for NAD systems as it is very common for point mutations to occur in many infectious agents (i.e. RNA viruses). Mutations, which may occur within the primer or probe sites can affect the efficiency of the assay and even invalidate the established performance characteristics. It is also advisable to regularly confirm the target sequence at the selected genomic regions for national or regional isolates of the infectious agents. This is especially true for the primer and probe sites, to ensure that they remain stable and the estimates of DSe for the assay are not compromised.

A similar situation may occur with incursion of new viral lineages into countries or regions where that viral lineage did not previously exist. In these circumstances, existing NAD assays that did not target these novel lineages may need to be modified to include primers or probes targeting these new analytes. The same would be true for typing sera used in virus neutralisation assays.

i) Technical modifications and comparability assessments

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 is done to determine if the relatively minor modifications of the assay affect the test results. Comparability can be established by running the modified procedure and original procedure side-by-side, with the same panel of samples in both, over several runs. The panel chosen for this comparison should represent the entire operating range of both assays. If the results from the modified procedure and originally validated method are determined to be comparable in an experiment based on a pre-specified criterion, the modified assay remains valid for its intended purpose. See Appendix 1.1.4.6 for description of experiments that are appropriate for comparability testing and Appendix 1.1.4.7 on reference sample panels.

~~A comparability assessment may or may not be adequate if the test is applied to a different sample matrix, e.g. validated on blood and used on another tissue in the sample species. Revalidation may be necessary if the test is designed for use in a new species.~~

ii) Biological modifications and comparability assessments

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 the test specimen itself (e.g. a change in tissue to be tested or perhaps testing of a different species altogether in an NAD system). It may include changes to reagents themselves (e.g. the substitution of a recombinant antigen for a cell culture derived antigen or one antibody conjugate for another of similar immunological specificity in an ELISA). The difficulty in making any modification or enhancement lies in determining whether the change requires a complete revalidation of the assay at both bench and field levels. At the very least, any modification would require that the appropriate Stage 1 'analytical requisites' be assessed. The more difficult decision relates to Stage 2 'diagnostic performance'. To assist here, the original (reference) assay should initially be compared to the modified (candidate) assay in a controlled trial using a defined panel of positive and negative diagnostic samples. See Appendix 1.1.4.6 for a description of comparability assessment using diagnostic samples. If the comparability assessment

does not suggest any significant change in diagnostic performance, the modified assay may be phased into routine use. If, on the other hand, significant differences are observed, the modified assay would require additional Stage 2 or field validation before being adopted.

iii) Replacement of depleted reagents

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

c) Enhancing confidence in validation criteria

Because many host variables have an impact on the diagnostic performance of assays, it is highly desirable over time to increase the number of reference samples from animals of known infection status. This improves the precision of the overall estimates of DSe and DSp, and may allow calculations of DSe estimates by factors such as age, stage of disease, and load of organisms. New data should be included annually in relevant test dossiers.

d) In house validation

Text under study.

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APPENDICES TO VALIDATION CHAPTER ~~– UNDER STUDY~~

Appendix 1.1.4.1. Development and optimisation of antibody detection assays

Appendix 1.1.4.2. Development and optimisation of antigen detection assays by immunological means

Appendix 1.1.4.3. Development and optimisation of Nucleic Acid Detection (NAD) assays

Appendix 1.1.4.4. Measurement ~~of~~ Uncertainty

Appendix 1.1.4.5. Statistical Approaches to Validation

Appendix 1.1.4.6. ~~Methods~~ Comparability of assays after minor changes in a validated test method

Appendix 1.1.4.7. Reference samples and panels – selection and use

ADVICE FOR MEMBER COMMENTS

Appendix Number and Title: 1.1.4.1. Development and optimisation of antibody detection assays

Country making the comments

Date

NB: The Biological Standards Commission is particularly interested in OIE Member Country opinion on this appendix (and its accompanying chapter and six other appendices): are the documents clear and useful? If not, how they could be improved?

It would be appreciated if the following guidance notes are followed in making a reply:

1. *Comments may be general or specific, but specific comments are usually more valuable. General comments should be such that some conclusion and action can be taken in response to them. For example, it is of little help to make statements such as "This test is no longer used in our laboratory", without indicating the reasons and what test is used instead.*

2. *Specific comments should be identified, by indicating the line number in the text, to facilitate the making of editorial changes.*

3. *The pointing out of typing or technical errors is welcome, but the correct word or figure should be indicated. For example, it is of little help to say that 0.8 M is too high, if the preferred value is not indicated.*

4. *Bear in mind that the introductory chapters are not intended to be exhaustive, and indeed none of the chapters can give a completely comprehensive cover of the subject, otherwise the* Terrestrial Manual *would be too long. However, assistance in indicating priorities is always helpful.*

5. *The* Terrestrial Manual *is intended for world-wide use. The chapters need to reflect the development of new technology, but the old established methods, usually requiring less sophisticated apparatus, have to be retained. New technology should not be described in detail until it has gained wide acceptance as a reliable method.*

General Comments

Specific Comments (*add continuation sheets if required*)
line:

APPENDIX 1.1.4.1.

DEVELOPMENT AND OPTIMISATION OF ANTIBODY DETECTION ASSAYS

INTRODUCTION

Detection of antibodies that are elicited in response to infectious agents or their components, constitute an indirect means of laboratory-based disease diagnosis. The most common antibody detection methods are classical virus neutralisation (*VNT*)*, enzyme-linked immunosorbent assay* (*ELISA*)*, haemagglutination inhibition* (*HAI*) *and the complement fixation test* (*CFT*)*. More recent novel methods include biosensors, bioluminometry, fluorescence polarisation, chemoluminescence and lateral flow devices also known as point of care or pen-side tests. Other immunological assays that use antibodies in antigen detection tests are described in Appendix 1.1.4.2.*

When considering a candidate assay type for disease diagnosis, one should include antibody detection assays because of their practicality, ease of sample collection and preparation, generally good diagnostic performance characteristics, suitability for automation (*high-throughput*)*, low cost and fast turn-around time. They are particularly useful for processing large numbers of samples in epidemiological and population studies, or for mass diagnosis and surveillance programmes. Antibody assays are also widely used for export, import and trade of animals, and still represent the majority of OIE recommended tests for international trade.*

A characteristic of antibody assays is their capacity to indicate prior exposure to an infectious agent in the absence of detectable organisms or their analytes. They are also adaptable to a variety of matrices, such as serum, plasma, whole blood, milk, lacrimal secretions and saliva. Immunoglobulin isotype or subclass-specific test systems may selectively target early or late immune responses, e.g. IgM and IgG, respectively. Specifically designed detection systems allow differentiation between responses to vaccine and field strains and are available as commercial kits, e.g. the detection of antibodies to classical swine fever virus in pigs. Competitive or blocking formats allow use of the same basic assay for a variety of animal species while other formats are species specific. Many types of chemical or physical indicators are used to indicate the presence of specific antibody in a specimen (*chromogens, fluorochromes, agglutinins, among many others*)*. Because of the large number of antibody detection methods available, it is not possible to describe the best practices for validation of each of these assay types in this Appendix. The most widely used antibody detection system, the ELISA, will therefore be used as an example for application of best practices in antibody assays. Most of the basic processes used to validate other types of assay systems will become evident by extension of those used to validate ELISAs.*

A. ANTIBODY DETECTION ASSAY DEVELOPMENT PATHWAY

1. Intended purpose(s) of the antibody assay

The first consideration in assay development is to clearly define the specific purpose and application of the test to be developed. Many decisions in developing assays will be based on these first considerations. For antibody detection assays (hereafter designated as “antibody assays” in this Appendix) such as ELISA, such knowledge will guide the selection of the most appropriate type of antibody detection system to achieve the intended purpose. Many factors related to the assay’s intended purpose, use, and suitability need to be taken into account (see Chapter 1.1.4/5., Section A.1.b. for additional prompts on fitness for use).

The six basic intended purposes for diagnostic assays are stated in Chapter 1.1.4/5., and listed in the footnote to Table 1. Because antibody assays have such a broad range of applications, and can be configured for very specific purposes, it is useful to consider and evaluate several parameters when establishing the specific purpose(s) for the candidate assay. Table 1 summarises a combination of antibody and antigen detection

possibilities for several assay characteristics that are common to all assay types. Consideration of these characteristics will provide guidance in establishing the specific purposes for which the candidate assay will be fit.

Note – The reader is advised to read Section B.4.a) on Programme implementation, below, as a primer for the following discussions. That Section describes the inter-relationships between diagnostic sensitivity and specificity, false positive and negative rates, and positive and negative predictive values. For a more in depth discussion of predictive values as a function of prevalence, see Jacobson, 1998.

Table 1. *Determinants of an assay's fitness for its intended purpose*

	Determinants of fitness for purpose						
Characteristics of assays	***1****		***2****	***3****	***4****	***5****	***6****
	a	b					
Test type (**Ab**/Agent)	**Ab**	**Ab**/Ag	**Ab**	**Ab**/Ag	**Ab**/Ag	**Ab**	**Ab**
DSe	+++	+++	+++	+++	+++	+	+
DSp	+	+	+	+	+++	+	+++
Positive predictive value (PPV)	+	+	+	+	+++	+	+
Negative predictive value (NPV)	+++	+++	+++	+++	+++	+	+++
Throughput capacity	+	+++	++	+	–	++	++
Turn-around time of test	+	+	+	+	+++	–	+
QA capability	+++	+++	+++	+++	+++	+++	+++
Reproducibility	+++	+++	+++	+++	+++	+++	+++
Repeatability	+++	+++	+++	+++	+++	+++	+++
Technical sophistication	Disease specific		→				
Interpretation skill	Disease specific		→				

Abbreviations: Test type: Ab = antibody detection; Ag = Antigen detection; DSe = Diagnostic Sensitivity; DSp = Diagnostic Specificity

Symbols: +++ = essential; + = of less importance; – = not important

*** Basic purposes for which an assay may be deemed fit:**

1. Demonstrate freedom from infection in a defined population (country, zone, compartment, herd; prevalence apparently zero). a) 'Free' with vaccination; b) Re-establishment of 'freedom' post-outbreaks
2. Certify freedom from infection or presence of the agent in individual animals or products for trade/movement purposes
3. Eradication of disease or elimination of infection from defined populations
4. Confirmatory diagnosis of clinical cases (includes confirmation of positive screening test)
5. Estimate prevalence of infection or exposure to facilitate risk analysis (surveys, herd health status, disease control measures)
6. Determine immune status in individual animals or populations (post-vaccination)

- **Purpose 1**

For disease freedom categories as given in purposes 1a and 1b (Table 1), antibody screening tests of high DSe are the tests of choice. As indicated in the purposes above, these tests would be applied to populations that have an apparent prevalence of zero. Tests of high DSe demonstrate low false negative (FN) rates and when applied to low prevalence populations, the NPV is at its highest level. However, DSe and DSp are usually inversely proportional and as such, a decrease in DSp will result in an elevated false positive (FP) rate. Other considerations, if this is to involve a continuous volume of surveillance samples, would include high throughput, low cost and technical simplicity. All screening test positive reactors should be subjected to some form of confirmatory testing to evaluate their true status. Confirmatory tests characteristically have high DSp and therefore a low FP rate. These tests are often more sophisticated and require enhanced interpretive skills.

If demonstration of freedom from infection is to be achieved after an outbreak, in which vaccination has been used for disease control, then screening of massive numbers of sera is often required. In addition to the considerations above, this also necessitates an antibody detection test which is able to distinguish between infected and vaccinated animals. At the same time an antigen or nucleic acid detection test may be warranted in some situations to prove that shedding and/or circulation of the infectious agent has ceased.

- **Purpose 2**

If the purpose is to qualify individual animals for international movement, antibody screening tests of high DSe are again the tests of choice. The same rationale as stated above applies with respect to the NPV. Again, all positive reactors will need to be subjected to some form of confirmatory testing to evaluate their true status. In cases where borderline positives are observed, it may be wise to request a repeat sampling of the animal(s) at a suitable time interval to ensure that herd/flock has not been very recently infected.

- **Purpose 3**

If the purpose of the test is the eradication of disease or elimination of infection from defined populations, antibody screening tests of moderate to high DSe are the tests of choice. However, the rationale is a bit different in that the testing will likely be done at herd or compartment level. At the beginning of the campaign, when the disease prevalence is high, moderate DSe and DSp are suitable as both FP and FN rates are less relevant at this juncture and a moderate level of error is tolerable. Depending on the nature of the disease and rapidity of spread, high throughput and fast turn-around-times may become critical. Usually decisions are made without confirmatory testing at this point.

In the latter stages of the campaign, a higher DSe is warranted as the FN rate becomes the more critical factor. Much like Purposes 1 and 2, positive reactors will need to be subjected to some form of confirmatory testing to evaluate their true status. In these latter stages, antibody detection tests are often applied in conjunction with antigen and/or nucleic acid detection systems to detect sub-clinical cases and possibly, latent carriers.

- **Purpose 4**

For the confirmatory diagnosis of clinical cases, antibody tests of high DSp are the tests of choice. In these cases, the idea is to minimise the FP rate and enhance the PPV of the test. As a general rule, infection is well established and the immune response is usually well underway. For some clinical cases, e.g. vesicular diseases in terrestrial animals, several tests may be required to rule out select pathogens that present similar clinical signs. In some cases, antigen and/or nucleic acid detection tests may be a better choice for confirmation of clinical cases provided that they offer a fast turn-around-time. A prime example would be highly pathogenic avian influenza infections where mortality may occur before an immune response is even detectable.

- **Purpose 5**

For estimates of prevalence of infection or exposure to facilitate risk analysis, e.g. for health surveys, herd health status and to monitor disease control measures, antibody tests of moderate DSe & DSp are the tests of choice. In general, this would balance both FN & FP rates and result in a more accurate estimate of the true prevalence of infection in the target population. However, if accurate estimates of both DSe and DSp have been established, statistical approaches can be used to minimise bias attributable to FN & FP rates (see Appendix 1.1.4.5 Statistical approaches to validation).

- **Purpose 6**

For the determination of the immune status in individual animals or populations, e.g. post-vaccination, antibody tests of high DSp are required. Such tests have very low FP rates and as such provide a high degree of confidence in the PPV of the result. For use in individual animals, the use of virus neutralisation (VN) tests in cell culture for the detection of vaccine-induced neutralising antibodies against rabies virus in dogs and cats would be a prime example of a test with high DSp used for expression of titres in international units. However, these tests are technically sophisticated, expensive to maintain and run, and require strict biosafety procedures. For larger volume applications, such as monitoring regional vaccination programmes, e.g. for Rinderpest, ELISA-based tests would be more applicable, given their simplicity, cost effectiveness and high throughput. The same DSp considerations should be applied to these types of tests.

The experience of laboratory diagnosticians is not only essential in the choice of an appropriate test that will achieve the desired purpose, but is also required to reliably determine the scientific limitations of an assay and practical considerations such as cost, equipment and reagent availability, throughput capacity of the laboratory and test turn-around-times.

2. Assay development – experimentation

a) Reference materials, reagents and controls

i) Test samples

Samples to be tested in antibody assays should be handled as described in Chapter 1.1.1. The sample matrix in which antibodies are usually detected is serum, but may also include plasma, whole blood, milk, lacrimal secretions and saliva.

ii) Reference Standards

Antisera directed against the reference strain of a pathogen are known as reference sera or reference standards (Wright *et al.,* 1993; Chapter 1.1.4/5., Section A.2.g; Appendix 1.1.4.7. Selection and use of reference samples and panels). Such sera containing antibody of known concentration/activity are useful in the initial development of an assay. For a number of OIE listed diseases, e.g. AI, FMD, CSF etc. international reference standards are available through OIE Reference Laboratories and Collaborating Centres. When not available from other sources, it may be necessary to produce in-house reference standards against which working standards (process or quality controls) are calibrated.

iii) Positive and negative reference panel

These sera, containing concentrations of antibody over the intended operating range, (also known as dynamic range) of the assay, should be used throughout the development and standardisation of an antibody assay. It is recommended that they be prepared in sufficient quantities so that they may be used in various aspects of validation. These samples should represent known infected and uninfected animals from the population that eventually will become the target of the validated assay. They should preferably be derived from individual animals, but they may represent pools of samples from several animals (Appendix 1.1.4.7. Selection and use of reference samples and panels).

iv) Monoclonal antibody reagents

The advent of monoclonal antibodies has greatly enhanced enzyme immunoassays. Whereas polyclonal anti-immunoglobulin conjugates are the staple of most indirect ELISAs, monoclonal conjugates can be directed to specific immunoglobulin isotypes. Depending on the immunoglobulin epitope targeted, many of these monoclonals can be effectively used to detect antibodies in related species, e.g. ruminants. Using monoclonal conjugates to either light or heavy chain epitopes can effectively modulate the DSp and DSe of the indirect ELISA.

Monoclonal antibodies are best known for their application in competitive or blocking ELISAs. In this case, the monoclonal specificity is directed to epitopes on the pathogen in question. Depending on the epitope targeted, the analytical specificity of the assay can be modulated in terms of inclusivity and exclusivity (see Chapter 1.1.4/5., Section B.2.b).

Monclonal antiobodies can also be used in sandwich ELISAs, either for trapping antigen to the plate or for subsequently detecting antigens that have been trapped. Depending on the size and complexity of the antigen in question, it is sometimes preferable to use a polyclonal antibody preparation for trapping as they generally contain antibodies of high binding affinity.

v) Antigens

Antigens used in ELISA's are of critical importance to diagnostic performance given a particular application. The choice of antigen greatly affects the inclusivity and exclusivity of the assay (see Chapter 1.1.4/5., Section B.2.b). Antigens expressing highly conserved epitopes, such as those found in some viral matrix or nucleoproteins, are generally useful in group specific assays, e.g. ELISA's for the detection of responses to all Influenza A viruses. Other antigen epitopes can be used to restrict detection to certain serotypes. The choice of antigen must be carefully researched and considered.

Crude antigen preparations, like cell lysates, used to be commonly used. However, antigens improved greatly as purification techniques advanced, e.g. affinity chromatography. Further improvements were achieved given the application of molecular cloning. Recombinant antigen technologies have greatly enhanced all aspects of ELISA performance, from analytical through diagnostic characteristics.

b) Design of test method

Designing the method

- Has the design been shaped by the intended purpose of the assay?
- What is the specific application?
- What are the types and statistically relevant numbers of samples to be tested? (See Appendix 1.1.4.5)
- Will the test be field or lab based?

i) Choice of test

In designing a test, its intended application will influence the choice of assay format that is best suited for the task. For example, if its use is primarily for surveillance, then the type of ELISA needs to be conducive to achieving high DSe, as described in the 'Purposes' above. It also should be analytically inclusive (Chapter 1.1.4/5., Section B.2.b) for

Aspects affecting choice of test

- Is the assay to be used for screening or confirmatory purposes, or both?
- Will it be used for one or more species? Which ones?
- Is the test intended for detection of early or late infection?
- Will the test be used for a virus, sero- or strain-specific assay?
- Will the assay be used to confirm sero-conversion after vaccination?
- Will it be a DIVA assay (differentiation of vaccinated from infected animals)?
- Will the test be applied to trade?

detection of all variants of, say, a type species. If, however, the screening assay's DSe is set so high that it generates many false positives, then a companion confirmatory test should also be considered at the same time. Many ELISA formats are available, each with their advantages and disadvantages that allow customisation of assays for very specific purposes (Table 2).

Important factors that influence the choice of an antibody assay format are availability of reagents and the operating range required for a particular assay. A limitation may be the unavailability of relevant antibody reagents for a particular format, e.g. competitive or blocking formats generally require antigen-specific monoclonal antibodies. Another example would be the need for an effective capturing antigen: a rather crude antigen may be acceptable for use in a sandwich-based ELISA screening assay, whereas a purified antigen would be necessary for a confirmatory assay. Other important considerations for choosing a particular ELISA format are which antibody isotypes, concentrations, avidities and antigenic specificities are diagnostically relevant. Which antigen, and in particular which epitopes are relevant. All will play a large role in selecting a particular type of ELISA (Table 2). If it is anticipated that the test will be used in different species, including wildlife, a competitive format may be useful.

Practical matters in selecting an assay format

- Is high-throughput essential? Will it be automated?
- What is the anticipated turnaround time? Is that suitable?
- What level of sophistication is needed to run the assay?
- What skills are required to interpret the test?
- Will that assay be feasible for use in my laboratory?
- Will it be easily transferrable to other laboratories?

Critical points to be addressed:

- Have you considered that concentrations of analyte in matrix significantly impact the lower limit of antibody detection and the operating range of the assay?
- Are the required antibody reagents (mono/polyclonal) available?
- Is available antigen sufficiently purified?
- Are reagents commercially available? If not, is it practical to produce them in-house?
- Are reference standard reagents available? If not, how are you going to resolve this deficiency? (see Chapter 1.1.4, section A.2.g)

Deciding on an assay format also requires that application of the assay be considered. Questions that should be addressed are detailed in the box under Section A.2.b.i., and practical questions in the boxes below Table 2. It is essential to deal with such questions at this point in assay development as they are essential to a positive outcome and application.

***Table 2* (*cont.*)** *ELISA formats: advantages and disadvantages**

Type of ELISA	*Advantage*	*Disadvantage*
Indirect – Ab detected by anti-species conjugate	Use and availability of high variety of antispecies-specific conjugates often targeting particular antibody sub-sets, such as anti IgM, IgG1, IgG2, etc. Wide use for screening large numbers of samples	Variation in degree of nonspecific binding in individual sera To compensate for this problem high starting dilutions are required This can lead to a decrease in DSe in comparison to competitive formats Can only be used for one or a few species at a time
Sandwich – Ag presented on a solid-bound-phase capture antibody	The capture antibody on the solid phase can help to orient the antigenic molecule, which improves the chance that the sample antibody will bind. Unpurified antigen preparations can be used because capture antibody selectively binds crude antigen. Pre-coating with capture antibody can reduce the potential for subsequent binding of nonspecific proteins during the test.	Antigens must have at least two antigenic sites or epitopes which limits this type to relatively large antigenic complexes or more complex proteins Size and spatial relationship of epitopes can affect the assay

***Table 2* (cont.)** *ELISA formats: advantages and disadvantages**

Type of ELISA	*Advantage*	*Disadvantage*
Competition (indirect and sandwich types) – Test antibody in sample mixed with pre-titrated detection antibody, then added to wells coated with capture antigen, either in direct or inhibition/blocking format.	Easy adaptation for use as antibody detection tests When highly specific MAbs are used the antigen does not have to be highly purified Can be used in different species including humans (WNV, AI, EI) and wildlife (FMD, Rinderpest), e.g. for which no conjugated antibodies exist Advantage of competitive sandwich type relies on antigen capture Sera can be tested in low dilutions without risk of interference due to non-specific ab binding. This may contribute to a higher sensitivity of this format Different antibody concentrations can be used to favour either analytical sensitivity or specificity. This is particularly relevant for assays using polyclonal ab which are much more affected through the use of different dilutions of sera	Generally more steps and more optimisation may be needed, e.g. pre-titration and optimisation for liquid and solid phase reagents. Higher level of technical sophistication required

* Primary source is Crowther (2001).

c) Proof of concept experiments (feasibility studies)

After choice of an ELISA format, initial experiments are designed to determine if the proposed assay is viable. A reference panel such as described in Section 2.a) iii) should be tested in the prototype assay. If a reference standard is to be used for normalisation of test data, it should be selected and incorporated at this point in assay development. To provide continuity in data assessment throughout, both the reference panel and any reference standards should be included in all remaining aspects of the validation studies. The reference panel used in the feasibility study should span the entire anticipated operating range of the candidate assay and be run in replicates as a quick check for repeatability.

Proof of concept
- Was the feasibility study conducted with at least 4 to 5 samples spanning the operating range of the assay?
- Did you include one or more reference standards if required for data normalization data
- Was separation of results between negative, low positive and high positive samples adequate?

The assay should achieve good separation in OD values, spanning the operating range of antibody activity. Adequate separation is particularly important between the negative and low positive samples. If the assay appears promising, optimisation is the next step.

d) Samples and data expression

i) Preparation and storage serum panels for optimisation studies

As stated, a best practice for antibody assays to select several (a minimum of four to five) serum samples that range from negative to high levels of antibodies against the infectious agent in question. These samples are initially used in experiments designed to demonstrate proof of concept. A large volume (e.g. a minimum of 10 ml) of each serum sample is acquired and divided it into 0.1 ml aliquots for storage at or below –20°C. One aliquot of each sample is thawed, used for experiments, and ideally then discarded. If it is impractical to discard the aliquot, it may be held at 4°C between experiments for up to about 2 weeks; however, there is a possibility of sample deterioration under these circumstances. Then, another aliquot is thawed for further experimentation. This method provides the same source of serum with the same number of freeze–thaw cycles for all experiments (repeated freezing and thawing of serum can denature antibodies so should be avoided). Also, variation is reduced when the experimenter uses the same source of serum for all experiments rather than switching among various sera between experiments. This approach has the added advantage of generating a data trail for the repeatedly run samples.

After the initial stages of assay validation are completed, one or more of the samples may be suitable as a reference standard for data expression and the entire panel may be used for repeatability

assessments both within and between runs of the assay (Jacobson, 1998). They may also serve as in-house working standards, i.e. quality or process controls given that their reactivity has been well characterised; such controls provide assurance that runs of the assay are producing accurate data (Wright *et al.,* 1993).

ii) Normalisation of results and their expression

An optical density (OD) reading in ELISA is a measurement of colour development that is a function the amount of antibody present in a sample. Because colour development is a function of a reaction of enzyme and substrate in the presence of a chromogen, results from day to day are subject to variation attributable to external factors such as temperature, reaction time, etc. Comparison of OD results for the same samples between runs of an assay in the same laboratory, or between laboratories, lacks precision. Therefore, OD results of test samples need to be adjusted as a function of the OD(s) of one or more reference standards included in each run of the assay. This process is known as "normalisation" of ELISA results (See Chapter 1.1.4/5., Section A.2.h for details. The method of normalisation and expression of data should be determined, preferably no later than at the end of the feasibility studies.

OD values may be expressed in several ways (Wright *et al.,* 1993). A simple method is to express all OD values as a percentage of a single high-positive serum control that is included on each plate. For such calculations, this control must yield results that are in the linear segment of the operating range of the assay. A more rigorous normalisation procedure is to calculate results from a standard curve generated by plotting observed OD values against concentration (or dilution) of antibody for several serum controls that span the range of antibody activity of the assay. It requires a more sophisticated algorithm, such as linear regression, log-logit, or 4 or 5 parameter logistic regression analysis, among others. This approach is more precise because it does not rely on only one high-positive control sample for data normalisation, but rather uses several serum controls, adjusted to expected values, to plot a standard curve from which the sample value is extrapolated. This method also allows for exclusion of a control value that may fall outside expected confidence limits.

e) Optimisation

For ELISAs, the most important variables that need to be optimised are concentration/dilution of antigen adsorbed to the solid phase, test serum working dilution, enzyme molarity, antibody-conjugate dilution, and substrate solution concentration. These are evaluated through checkerboard assessments (each variable compared against all other variables within one run of an assay that is repeated several times). Other variables that need consideration are pH and ionicity of reagents, molecular factors such as valency and epitope density of antigens, isotype of targeted antibody and antibody affinity. Precision of test results can be graphically depicted or expressed numerically by various statistical methods (Crowther, 2001). ELISA studies require that instrumentation (plate washers and readers, etc.) must be properly calibrated prior to use – part of the laboratory's quality control program.

Optimisation and standardisation

- Have all critical reagents been run against each other in checker board titrations?
- Did you find optimal concentration/dilutions for each reagent?
- Did you incorporate quality or process control procedures and reagents?
- Did you incorporate methods for normalization of test data?

f) Inhibitory factors in sample matrix

Although ELISA antibody detection systems are rather resistant to inhibitory factors, Chapter 1.1.4/5., Section A.2.e, and Greiner *et al.* (1997) provide descriptions of the type of inhibitors that could affect the assay. These references should be reviewed carefully to assure that all inhibitory factors are accounted for and controlled.

g) Calibration to reference standard sera

If international, national, or other-source reference sera are available, the assay should be calibrated to match the analytical sensitivity in terms of the metrological units ascribed to the calibration sera (Wright, 1998).

B. ASSAY VALIDATION PATHWAY

1. Stage 1 – Analytical performance characteristics

a) Repeatability

Analytical performance characteristics

- Has repeatability been established for a range of positive and negative samples within and between runs of the assay
- Have upper and lower control limits of the assay been established
- Have you defined ASe and ASp for this assay?
- Does the candidate assay compare favourably with a standard test method, based on objective quantitative and qualitative criteria?

Repeatability is the level of agreement between results of replicates of a sample, both within and between runs of the same method in one laboratory. The same or similar panel of samples used in the feasibility study is adequate. No less than three (preferably 5) samples covering the operating range of the assay, and of sufficient quantity for at least 20 runs of the assay over several days. Specifics of how the samples should be prepared and handled are provided in Appendix 1.1.4.7 and Chapter 1.1.4/5., Section B.2.a. It is valuable to include at least one reference sample in an indirect ELISA (a positive serum control) to which the test samples can be normalised by per cent of the positive control. The within run variation can be determined by the mean OD and coefficient of variation (CV) of the replicates of each sample. The CV should not exceed about 15% (with the possible exception of negative and very low positive samples which may have higher (and meaningless) CVs). If all of the samples have previously been calibrated to reference standards, and their expected ODs are thus known, the observed ODs for each sample in each run can normalised as a function of their expected ODs in linear regression analysis. This provides a correlation coefficient as evidence of closeness of fit to the expected value, and allows for normalised values to be plotted in control charts (Crowther, 2001).

b) Analytical specificity (ASp)

ASp is determined by testing sera from animals that are known to have been infected/exposed to all species/strains that the test should detect (Appendix 1.1.4.7. Group B a). For details on inclusivity, exclusivity and selectivity as sub-elements of ASp, see Chapter 1.1.4/5., Section B.2.b. Cross reactivity with sera from animals infected with related species is used to establish the desired discriminatory capacity of the test. ELISAs are also subject to false positive results attributable to exogenous factors, such as non-specific binding of serum or conjugate to the plastic surface that may require use of blocking agents. Care must be taken to eliminate this source of error.

c) Analytical sensitivity (ASe)

ASe is synonymous with the lower limit of detection (LOD) of antibody concentration in a sample. The different types of antibody assays vary considerably in their inherent limit in antibody detection. For instance, LODs for eight different types of antibody assays range from 1000 ng/ml (radial immunodiffusion) to 0.01 ng/ml (chemiluminescence) (Nielsen *et al.,* 1996). LODs are usually determined by endpoint dilution in which replicates (preferably 10) of each dilution in a $\log_2$ dilution series are run in the assay. The large number of replicates allows for a more precise determination of the dilution at which the antibody is no longer detectable. Accordingly, the last dilution in which at least 50% of its replicates are positive constitutes the LOD in the assay (see Methods comparison studies, Appendix 1.1.4.5).

d) Standard test method comparison with the candidate test method

The candidate test method should be run in parallel with an OIE or other accepted reference test method, using the same panel of samples on both, to determine whether the candidate method exhibits the same quantitative and qualitative characteristics as the standard method. Favourable comparability lends strength to the probability that the candidate method will be a successful substitute for the reference method (see also Methods comparison studies, Appendix 1.1.4.5).

2. Stage 2 – Diagnostic performance characteristics

Diagnostic sensitivity (DSe) and specificity (DSp) are the primary performance indicators of the validation process. Antibody assays are subject to the same general procedures to achieve estimates of DSe and DSp as required of all other assay types (see Chapter 1.1.4/5. Section B.3 for essential details). The number of samples needed to establish these estimates for a particular antibody assay require a sampling design that considers many variables. This includes creation of a sample panel that is tailored particularly for the intended purpose of the assay (e.g. a screening versus confirmatory test). It also requires predetermined desired levels of DSe and DSp (indicating acceptable levels of false negative and false positive results), allowable error in the estimates of such DSe and DSp, and the confidence level required for these estimates.

The number of animals required to establish acceptable DSe and DSp estimates are a function of the level of confidence desired in DSe and DSp estimates and the accepted allowable error. For instance, for a pathogenic disease like FMD, it is necessary to reduce the likelihood that infected animals will be misclassified as uninfected, which reduces allowable error in the test result which, in turn, increases the number of samples needed to establish a high level of confidence in the DSe estimates. Alternatively, for a confirmatory assay it is desirable to reduce the likelihood that uninfected animals will be classified as infected. A high DSp is then desired with minimal allowable error, requiring a larger sample size of uninfected animals. All of these general issues related to sample size, confidence intervals and allowable error in the DSe and DSp estimates are described in Chapter 1.1.4/5., Section 3, with additional detail and tables of sample numbers required available elsewhere (Jacobson, 1998).

It is often challenging to obtain a sufficient number of well characterised sera to achieve estimates of DSe and DSp that are sufficient for the intended purpose of the assay. Initially, it may be a compromise between what is statistically meaningful and practically feasible, resulting in an assay that is provisionally recognised (Chapter 1.1.4/5., Section 2.g). However, over time, with accumulation of more well characterised samples, the estimates of DSe and DSp may be strengthened (see Section 5.d below).

a) The challenge in establishing accurate estimates of DSe and DSp for antibody assays

Antibody assays undergoing validation pose unique problems when attempting to assemble known positive and known negative samples in sufficient quantity to establish assay performance characteristics. Antibody is an indirect indicator of the presence of, or prior exposure to, an infectious agent or its components. Inferences from detection of antibody (or the lack thereof) depend on the host's qualitative and quantitative responses to the organism. Factors that affect the concentration and composition of specific antibody in serum samples are inherent to the host (e.g. age sex, breed, nutritional status, pregnancy, immunological responsiveness) or acquired (e.g. passively acquired antibody, or active immunity elicited by vaccination or infection). Theoretically, samples from animals that represent all of these variables should be included in the panels used for establishing DSe and DSp estimates. Clearly, this becomes a daunting, if not impossible task. To surmount this problem, the initial sample panels should be representative of the majority of animals in the target population to achieve initial estimates of DSe and DSp. In reality, it is necessary to enhance DSe and DSp estimates after the assay has been implemented as more well characterised samples become available (See Section 5.d, below).

Because it is often desirable to stretch the application of antibody detection assays to a huge number of animals spanning large geographical areas (e.g. as in screening assays for an entire continent), assembly of fully representative sample panels for such a large diagnostic window of variables may be nearly impossible. A useful alternative is to first establish DSe and DSp estimates for a rather homogeneous population of animals. If the assay is destined for use in disparate populations of animals, which may harbour a different infectious agent profile (with possibility of cross reactions not seen in the original targeted population), a reassessment of DSe and DSp may be necessary, drawing from data acquired using new sample panels that are representative of the population(s) targeted.

Diagnostic performance characteristics

- Are the criteria used to determine the positive and negative reference populations legitimate?
- Do the reference samples fully represent the population targeted by the assay?
- Were there difficulties in obtaining a sufficient number of samples? If so, how was the problem addressed?

b) Reference animal populations

i) Animals of "known infection status"

Reference animals of known infected and known uninfected status are the ideal source of samples for determining DSe and DSp. However, such samples are rare and difficult to establish. The most familiar term for reference animals or samples used in establishing DSe and DSp is the so called "gold standard", a misnomer commonly used to classify almost any reference animal as infected/exposed or uninfected, with samples from such animals classified as positive or negative (see Chapter 1.1.4/5., Section 3.a–c).

Assay developers should be aware of the advantages, and particularly the pitfalls, associated with various methods that are used to classify reference animals as infected or uninfected. The samples from such animals are deemed either positive or negative, and collectively become the *reference standard* upon which the candidate assay's DSe and DSp are based. It is, therefore, crucial to carefully consider the validity of various reference standards as exemplified in the following four examples:

- ***An unequivocal reference standard*: *presence of the agent in the host or evidence of definitive histopathology***. If an infectious agent or definitive histopathological criterion is detected in an animal, this generally constitutes an unequivocal reference standard for that animal. Serum samples derived from such animals usually are considered to be unequivocal serum

reference standards for determining DSe and DSp of the candidate assay. However, such samples may have their limitations. At the population level, a pathogen may be unequivocally present in some animals, but if the serum sample was taken from the animal early in the infection process, the immune response may not yet have produced detectable antibody. In this case, such serum samples used as reference standards would have been FN for the subset of animals in an early stage of infection. In contrast, for more chronic types of infection, using only reference animals that have confirmatory culture or histopathology may produce higher estimates of DSe than are realistic for the population targeted by the assay because the immune response will always be well established.

- ***A composite reference standard: verification of uninfected or unexposed animals***. This standard is achieved by selecting reference animals from geographical areas where herd histories, clinical profiles, prior testing results and other parameters provide evidence suggesting the absence of the pathogen, and thus no specific host antibody response to the pathogen targeted by the candidate assay. These types of reference materials, their strengths and limitations are described elsewhere (Jacobson, 1998), and must be considered carefully when using samples from such sources for establishing DSe and DSp for a candidate assay.

- ***A relative reference standard*: comparative serology.** This standard is characterised by reference animals that have been classified for their infection status by comparison with the tests results of another serological assay on the same samples. It often is the only practical source of reference material available for evaluation of a new serological test. If results of such a reference test are chosen as the standard for determining diagnostic performance characteristics of the candidate assay, the resultant estimates of DSe and DSp are useful only insofar as the reference test has documentable, established and acceptable performance characteristics. A deficiency of relative reference standards is that they have their own established levels of FP and FN test results, which are sources of error that will be compounded in estimates of DSe and DSp for the new assay. Generally, however, the use of other well described test methods is regarded as good practice to determine the status of reference animals, but only if the inherent bias introduced by the relative reference standard is accounted for (World Organisation for Animal Health [OIE], 2008).

- ***An adjunct reference standard*: experimental infection or vaccination** (see Chapter 1.1.4/5., Section 3.c for significant limitations of this type of standard). In some cases, the only way to obtain positive samples is by experimental infection. This approach is highly suitable to model the dynamics of the infection and to determine the 'diagnostic window' with the new assay. For example, it is possible to get estimates of the time interval between exposure to a pathogen and when antibody is first detectable, or when 25, 50, 75 and 100% of the infected animals return a positive test result. Nevertheless there are pitfalls in use of time-series data that must be avoided. Data representing repeated observations from the same animals cannot be used in calculation of DSe and DSe because the statistical models used to establish DSe and DSp require require independent observations (only one sample from each anima)l. For statistically legitimate time-course studies, or when single samples are used from each of many experimental animals, the strain of cultured organism, route and dose of exposure, infection with other related, cross-reactive and non-related, non-cross-reactive organisms are variables which may produce quantitatively and qualitatively atypical responses which are not found in natural infections in the target population. Experimental conditions typically lead to an overestimation of sensitivity and specificity for example by artificially high challenge doses and by using specific pathogen free animals as negative controls.

  The time point of sample collection (days post infection) must be indicated. Sources and history of experimental animals should be described. The validation should not solely be based on experimental animals for by definition, it is nearly impossible for them to fully represent natural populations of animals subject to pathogens by natural exposure.

ii) Latent-class models for sample selection

For a discussion of this approach to sample selection, see Chapter 1.1.4/5., Section 3.b.ii and Statistical approaches to test validation Appendix 1.1.4.5.

c) Threshold (cut-off) determination

The procedures for establishing the cut-off between negative and positive results of antibody assays are as described in Chapter 1.1.4/5., Section 3.d.

3. Stage 3 – Reproducibility and augmented repeatability estimates

Reproducibility is the measure of precision of an assay when used in several laboratories located in distinct regions or countries using the identical assay (protocol, reagents and controls) to test the same panel of samples.

Reproducibility assessments for antibody assays are not uniquely different from similar assessments for any other type of assay. Therefore the reader is directed to Chapter 1.1.4/5., Section 4, for details on reproducibility analysis and for reference samples and panels to Appendix 1.1.4.7.

4. Stage 4 – Programme implementation

a) Interpretation of results

Best practices for programme implementation are general to all assays types (Chapter 1.1.4/5., Section B.5). However, since ELISA is often the assay of choice for surveillance programs to affirm absence of disease, or for eradication of disease or elimination of infection from defined populations, the issue of false positive results can be a significant problem even if the diagnostic specificity is very high.

A common misperception is that a test with 99% DSp and DSe will only mis-classify animals as false positive (FP) or false negative (FN) 1% of the time. Actually, the FN and FP rate varies depending on the prevalence of infection in the targeted population. False positive reactions in a disease eradication campaign can vary significantly from the beginning of the campaign when prevalence is relatively high (for example, 10%) to near the end of the campaign when it has dropped to 0.1%. The predictive values of test results then become very important. Predictive values are probabilities that a test result is truly positive or truly negative. In our example using an assay with 99% DSe and DSp for testing a population of animals with a 10% prevalence of disease, the predictive value of a positive test result (PPV) is 91.7, meaning that there is a 91.7% probability that the animal is truly infected. The predictive value of a negative test result (NPV) is 99.9. When the prevalence drops to 5%, the PPV and NPV are 83.9 and 99.9, respectively. However, if the prevalence drops further to 0.1%, by successfully removing infected animals from the population, the same test will produce a PPV of 9% and a NPV of 99.9%, meaning that there is only a 9% chance that a positive test result is detecting a truly infected animal (of 1000 animals tested, only about 1 in 10 positive test results is indicative of an infected animal – the other 9 are false positive). So, if the test is intended for the purpose of eradication of a disease or elimination of infection from a population, the test developer is advised to consider moving the assay to a second cut-off that yields a higher DSp late in the campaign to reduce the probability of false positive reactions. It is instructive to examine a predictive value chart for assays of varying DSe and DSp, to visualise the effects of reduced prevalence on predictive values of an assay (Chapter 1.1.4/5., Table 2, and Jacobson, 1998).

5. Monitoring assay performance

a) Monitoring the assay

Once the assay is in routine use, internal quality control is accomplished by consistently monitoring the assay using quality control charts for assessment of repeatability and accuracy. Charts representing at least 30 runs will reveal trends or shifts in values of controls and standards. Lines representing the mean value of a control sample in at least 30 runs, plus/minus 3 standard deviations, are useful decision criteria for inclusion or exclusion of a run of the assay. The run is rejected if one control/standard exceeds ± 3 STD or if 2 controls (or more) exceed ± 2 STD (Crowther, 2001). Decision criteria may need to be customised for a given assay because of inherent differences between assays attributable to the host pathogen system. Appendix 1.1.4.4 provides an example of how to apply Measurement Uncertainty for an antibody ELISA using a positive internal control sample.

Monitoring assay performance

- Has the purpose of the assay changed?
- Has the epidemiology of the disease in question changed, e.g. prevalence, new serotypes or strains, etc.?
- Have critical reagents been changed, and if so, was comparability of the new reagents assessed?
- Are performance indicators included in day to day use of the assay (control charts, basic statistics)?
- Are upper and lower limits in control charts updated periodically as more experience with the control samples is achieved?
- Are test panels shared with other laboratories to assess reproducibility?
- Is proficiency testing included as part of continuing evaluation of the assay?

Reproducibility of test results or External Quality Assurance between laboratories should be assessed at least once per year if not twice and is an essential requirement of ISO 17025 accredited laboratories (World Organisation for Animal Health [OIE], 2008). Membership in a consortium of laboratories that are interested in evaluating their output is valuable.

b) Minor modifications of the assay – replacement of depleted reagents

When quality or process control samples are nearing depletion, it is essential to prepare and repeatedly test the replacement samples. The replacement samples should be included in at least 10 routine runs of the assay, with their results normalised against the existing reference standard. The activity of the replacement control should be comparable to the replaced control. If the reference standard requires replacement, care must be taken to select a replacement that matches all of the original serum characteristics as closely as

possible, thus allowing use of the replacement to normalise test results with comparable outcomes (see also Appendix 1.1.4.5.).

When other reagents such, such as antigen for capture of antibody, must be replaced they should be produced or procured using the same protocols or criteria as used for the original reagents. They need to be assessed using sera from routine submissions in 5–10 parallel runs that include the current and the new reagent(s). A panel of representative samples such as a proficiency panel, is also a useful tool for assessing the comparability of the reagents (Appendix 1.1.4.7).

c) Major modifications of the assay – changing to a new ELISA type

If the assay is to be changed from, say, a sandwich ELISA to a competitive format, the assay will require revalidation because of the many variables that may affect the performance characteristics of the assay. For an assay considered for implementation in another geographic region, e.g. from the northern to the southern hemisphere, it is essential to revalidate the assay by subjecting it to sera from populations of animals that reside under local conditions. Evaluation of reference sera that represent those populations is done by using stages 3–5 in Figure 1 in Chapter 1.1.4/5. It is the only way to assure that the assay is valid for populations that are of different composition compared with the original population targeted by the assay.

d) Enhancing confidence in validation criteria

Due to the extensive set of variables that have an impact on the performance of serodiagnostic assays, it is useful to expand the number of reference sera when possible, recognising the principle that error is reduced with increasing sample size. An expanded reference serum bank should be accumulated with well characterised sera, and used periodically to update estimates for DSe and DSp for the population targeted by the assay.

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ADVICE FOR MEMBER COMMENTS

Appendix Number and Title: 1.1.4.2. Development and optimisation of antigen detection assays

Country making the comments

Date

NB: The Biological Standards Commission is particularly interested in OIE Member Country opinion on this appendix (and its accompanying chapter and six other appendices): are the documents clear and useful? If not, how they could be improved?

It would be appreciated if the following guidance notes are followed in making a reply:

1. *Comments may be general or specific, but specific comments are usually more valuable. General comments should be such that some conclusion and action can be taken in response to them. For example, it is of little help to make statements such as "This test is no longer used in our laboratory", without indicating the reasons and what test is used instead.*

2. *Specific comments should be identified, by indicating the line number in the text, to facilitate the making of editorial changes.*

3. *The pointing out of typing or technical errors is welcome, but the correct word or figure should be indicated. For example, it is of little help to say that 0.8 M is too high, if the preferred value is not indicated.*

4. *Bear in mind that the introductory chapters are not intended to be exhaustive, and indeed none of the chapters can give a completely comprehensive cover of the subject, otherwise the* Terrestrial Manual *would be too long. However, assistance in indicating priorities is always helpful.*

5. *The* Terrestrial Manual *is intended for world-wide use. The chapters need to reflect the development of new technology, but the old established methods, usually requiring less sophisticated apparatus, have to be retained. New technology should not be described in detail until it has gained wide acceptance as a reliable method.*

General Comments

Specific Comments (add continuation sheets if required)
Line

APPENDIX 1.1.4.2.

DEVELOPMENT AND OPTIMISATION OF ANTIGEN DETECTION ASSAYS

INTRODUCTION

The detection and identification of an agent is confirmatory evidence of either infection with or disease caused by a particular pathogen. There are many different direct and indirect test methodologies available. Classical direct[1] *agent detection assays include electron microscopy, light microscopy* (*e.g. observation of unique histopathological or pathognomonic features, identification of parasites* in situ*, etc.*)*, virus isolation, bacterial culture and parasitic digestion techniques. Many direct techniques require secondary procedures to assist in the characterisation and identification of these agents* (*e.g. haemagglutination inhibition or virus neutralisation tests, special bacteria or fungal stains, etc.*)*. Representatives of indirect agent and in particular, antigen* (*Ag*) *detection tests include enzyme linked immunosorbent assays* (*ELISA*), *immunofluorescense or immunoperoxidase assays, Western blotting techniques, micro-arrays, fluorescence activated cell sorting* (*FACS*) *and biosensors. Often direct and indirect methods are used in tandem, for example a direct technique may be used to isolate, enrich and/or extract the organism followed by indirect techniques to characterise and identify the agent.*

Practical matters in selecting an assay format

- Is high-throughput essential? Will it be automated?
- Will the test be used as a herd test or for testing individuals?
- What is the anticipated turnaround time? Is that suitable?
- What level of sophistication is needed to run the assay?
- What skills are required to interpret test results?
- Will the assay be feasible for use in my laboratory?
- Will it be easily transferrable to other laboratories?

The examples given above vary widely in terms of laboratory requirements. Cost, laboratory infrastructure, biocontainment/safety, technical sophistication, interpretation skills, turn-around time, throughput capacity, diagnostic performance characteristics, repeatability and reproducibility are important parameters which need consideration when selecting the most appropriate assay. They also vary with respect to suitability for different diagnostic applications.

In contrast to serological or antibody detection tests, Ag detection tests depend heavily on the clinical onset and pathogenesis of the disease and the concentration of the pathogen in certain tissues and/or fluids. Successful diagnosis depends on appropriate timing and selection of sampling site (*affected tissue/lesions, scrapings, swabs, blood, other body fluids*) *and specimen integrity during transport. For certain applications, the testing of individual animals and/or samples may be appropriate* (*e.g. confirmatory testing*)*; while for other purposes* (*e.g. screening*)*, pooling of animals may be efficient and effective. Selection of the appropriate specimen requires good understanding of the disease and the effect of the sample matrix on the pathogen* (*e.g. cloacal or tracheal swabs for avian influenza*)*.*

Nucleic acid detection (*NAD*) *tests are increasingly replacing classical antigen detection systems. For development and optimisation of NADs, please refer to Appendix1.1.4.3. Although these NAD tests may seem to be the tests of choice for many applications, they are not always the most practical or efficient, given certain situations. In most cases it is still necessary and prudent, at least for an index case, to culture the agent on selective media or in susceptible cell lines or eggs to*

1 Definitions: In the context of direct and indirect detection of an analyte, the term “direct” is relevant to the organism or its antigens, e.g. direct detection by microscopy. The term “indirect” is relevant to the host response to the organism, (e.g., indirect detection inferred by detection of antibodies to the organism). In the context of diagnostic methods that are used to detect infection, microscopy is a means of direct observation of the organism, whereas using a reagent that infers the presence of the agent (such as an enzyme conjugated to a purified antibody that is specific for the agent) is an indirect method.

facilitate further characterisation and identification. While genotyping is an important consideration, especially in molecular epidemiology, other means of agent characterisation such as serotyping, pathotyping or biotyping are also important. Cultured and preserved agents have tremendous historical value and are also an important source of reference materials.

Due to its worldwide application, the Ag ELISA is used as an example for application of best practices in antigen detection assays in this appendix. Most of the basic processes used to validate other types of antigen detection assays will become evident by extension of those used to validate ELISAs. Because of the many conceptual similarities between antigen and antibody detection assays this appendix frequently cross-references to the antibody detection Appendix 1.1.4.1.

A. ANTIGEN DETECTION ASSAY DEVELOPMENT PATHWAY

1. Intended purpose(s) of the antigen assay

The intended purpose of the test is a key factor which will guide decisions in the selection and early development of the candidate assay. Given the OIE-defined 'fitness for purpose' categories in Table 1, Ag detection systems may be appropriate for certain applications. Support for disease eradication or surveillance programmes generally require testing of high numbers of samples, with an emphasis on diagnostic sensitivity and throughput capacity. In contrast, confirmation of clinical cases does not entail high numbers to be tested, but diagnostic specificity and turn-around-times become very important. At the outset, the questions posed in the text box, above, should be carefully considered.

Table 1. *Determinants of an assay's fitness for its intended purpose*

	Determinants of fitness for purpose						
Characteristics of assays	***1****		***2****	***3****	***4****	***5****	***6****
	a	b					
Test type (Ab/Agent)	Ab	Ab/**Ag**	Ab	Ab/**Ag**	Ab/**Ag**	Ab	Ab
DSe	+++	+++	+++	+++	+++	+	+
DSp	+	+	+	+	+++	+	+++
Positive predictive value (PPV)	+	+	+	+	+++	+	+
Negative predictive value (NPV)	+++	+++	+++	+++	+++	+	+++
Throughput capacity	+	+++	++	+	–	++	++
Turn-around time of test	+	+	+	+	+++	–	+
QA capability	+++	+++	+++	+++	+++	+++	+++
Reproducibility	+++	+++	+++	+++	+++	+++	+++
Repeatability	+++	+++	+++	+++	+++	+++	+++
Technical sophistication	Disease specific		→				
Interpretation skill	Disease specific		→				

Abbreviations: Test type: Ab = antibody detection; Ag = Antigen detection; DSe = Diagnostic Sensitivity; DSp = Diagnostic Specificity
Symbols: +++ = essential; + = of less importance; – = not important

*** Basic purposes for which an assay may be deemed fit:**

1. Demonstrate freedom from infection in a defined population (country, zone, compartment, herd; prevalence apparently zero). a) 'Free' with vaccination; b) Re-establishment of 'freedom' post-outbreaks
2. Certify freedom from infection or presence of the agent in individual animals or products for trade/movement purposes
3. Eradication of disease or elimination of infection from defined populations
4. Confirmatory diagnosis of clinical cases (includes confirmation of positive screening test)
5. Estimate prevalence of infection or exposure to facilitate risk analysis (surveys, herd health status, disease control measures)
6. Determine immune status in individual animals or populations (post-vaccination)

- **Purpose 1**

For disease freedom categories as given in purposes 1a and 1b (Table 1), antibody screening tests of high DSe are usually the tests of choice provided that the immune response is a significant feature of infection. However, there may be certain disease situations where the humoral immune response can be misleading and pathogen detection may be more appropriate (e.g. mycobacterial or trypanosomal infections). In these cases, Ag detection tests may be more effective. Much like antibody screening tests, an antigen screening test should demonstrate a high DSe. Tests of high DSe demonstrate low false negative (FN) rates and when applied to low prevalence populations, the NPV is at its highest level. As DSe and DSp are usually inversely proportional, a decrease in DSp is likely to result in an elevated false positive (FP) rate. Therefore, all screening test positive reactors should be subjected to some form of confirmatory testing to evaluate their true status. Confirmatory tests characteristically have high DSp and therefore a low FP rate. These tests are often more sophisticated and require enhanced interpretive skills.

If demonstration of freedom from infection is to be achieved after an outbreak, with or without vaccination, then screening of massive numbers of samples is often required. When antibody assays may be of limited value, antigen or nucleic acid detection tests may be warranted to prove that shedding and/or circulation of the infectious agent has ceased.

- **Purpose 2**

If the purpose of the test is the eradication of disease or elimination of infection from defined populations, antigen screening tests, like antibody screening tests, of moderate to high DSe, are the tests of choice. However, the rationale is a bit different in that the testing will likely be done at the herd or compartment level. At the beginning of the eradication campaign, when the disease prevalence is high, moderate DSe and DSp are suitable as both false positive (FP) and FN rates are less relevant at this juncture and a moderate level of error is tolerable. Depending on the nature of the disease and rapidity of spread, high throughput and fast turn-around-times may become critical. Usually decisions are made without confirmatory testing at this point.

In the latter stages of the campaign, a higher DSe is warranted as the FN rate becomes the more critical factor. Much like Purpose 1, positive reactors will need to be subjected to some form of confirmatory testing to evaluate their true status. In these latter stages, antigen and/or nucleic acid detection systems are critical in the detection of sub-clinical cases, shedders and possibly, latent carriers.

- **Purpose 3**

Although antibody tests of high DSp are often the tests of choice for confirmatory diagnosis of clinical cases, for some confirmatory diagnoses, antibody tests may not be the tests of choice, especially if clinical signs appear before an immune response is mounted. A prime example would be highly pathogenic avian influenza infections where mortality may occur before an immune response is even detectable. Antigen and/or nucleic acid detection tests are usually a better choice for confirmation of clinical cases provided that they offer a fast turn-around-time. In these cases, the idea is to maximise DSp and thereby minimise any potential FP reactions. For some clinical cases, e.g. vesicular diseases in terrestrial animals, several tests may be required to quickly rule out select pathogens that present similar clinical signs. In this category of test, fast turn-around-times are extremely critical in identifying potential outbreaks.

- **Purpose 4**

For estimates of prevalence of infection and/or shedding to facilitate risk analysis, e.g. for health surveys, herd health status and to monitor disease control measures, antigen detection tests of moderate DSe and DSp are the tests of choice. In general, this would balance both FN and FP rates and result in a more accurate estimate of the true prevalence in the target population. However, if accurate estimates of both DSe and DSp have been established, statistical approaches can be used to minimise bias attributable to FN and FP rates (see Appendix 1.1.4.5. Statistical approaches to validation).

2. Assay development – experimentation

a) Reference materials, reagents and controls

i) Test samples

Samples required for antigen detection assays should be handled as described in Chapter 1.1.1 of the OIE *Terrestrial Manual*. Sample matrixes for antigen detection assays can be very heterogeneous (e.g. blood, faeces, milk, skin, semen, saliva, blisters, vesicles or swabs from affected tissues such as oropharynx (Probang), trachea, genitals, cloaca, oesophagus, etc.). The ideal specimen is the one which is easy to obtain and with a high concentration of the analyte. In many cases blood or swabs are the specimens of choice but depending on the pathogen, other tissues or fluids are needed, e.g. skin, organs such as brain (rabies, transmissible spongiform encephalopathies [TSE]), lymphatic organs, such as spleen and lymph nodes (CSF), kidney, liver, heart, parts of respiratory tract (avian influenza), digestive tract (parvovirus), milk (enzootic bovine leukosis), feaces, semen, saliva, etc.

As stated in Chapter 1.1.1, the usual considerations apply to limit bacterial and fungal contamination of specimens. The use of preservatives and fixatives is not usually recommended and samples should be sent with minimal delay and under refrigeration to a diagnostic laboratory. During transport and storage it is important to be aware of the physical and chemical requirements of the pathogen (e.g. FMD virus is highly labile at low pH and requires equal amounts of glycerol and phosphate buffer to maintain a pH over 7).

If samples are to be tested as pools, experiments need to be undertaken to prove that the assay is fit for that purpose (e.g. that the ASe is sufficiently high to detect one infected animal in a in pool of 5, 10, 50 or more uninfected animals).

ii) Reference Standards

See Appendices 1.1.4.1. Development and optimisation of antibody detection assays, and 1.1.4.7. Selection and use of reference samples and panels.

iii) Positive and negative reference panels

Samples, containing concentrations of antigen over the intended operating range (also known as dynamic range) of the assay, should be used as controls during the development and standardisation of an antigen detection assay. They may be obtained from field specimens or produced in the lab as spiked samples (see Appendix 1.1.4.7. Selection and use of reference samples and panels). Negative samples should be obtained from known uninfected animals and this same matrix should be used to prepare spike samples.

iv) Purified and crude antigens for antibody production

In general, antigens to be used for the production of immunological reagents should be as natural as possible in terms of conformation to ensure that the presentation of epitopes mimics the orientation on the live organism. Therefore, isolation and/or purification methods used should preserve the antigenic integrity of the agent as much as possible.

For very large pathogens such as poxviruses, bacteria and protozoal parasites, protein microarrays may be useful to identify and select antigens which elicit strong immune responses.

v) Monoclonal and polyclonal antibodies for indirect antigen detection assays

Monoclonal antibodies demonstrate unique analytical specificities and are highly useful in the detection of agent-specific epitopes at the group, strain or sub-strain levels. As such, they need to be considered very carefully with respect to the application at hand and the desired inclusivity and exclusivity of the assay. Polyclonal antibodies, by nature, tend to demonstrate a broader range of specificities. Purified or semi-purified polyclonal antibodies are often the reagent of choice for trapping complex antigens because they usually demonstrate higher affinities than there monoclonal counterparts.

b) Design of test method

i) Choice of test

The prologue to a proper test design requires careful consideration of many variables in the context performance requirements. The choice of assay must be coupled with its intended application, which usually necessitates consensus between the assay developer, statisticians, and other stakeholders such as epidemiologists and regulatory bodies. If the purpose is to develop a screening test (e.g. during a post-outbreak surveillance period) the emphasis will be on high DSe, high throughput, low cost, technical simplicity, low interpretative skill, etc. If the purpose is to develop a confirmatory test (e.g. for the confirmation of clinical cases or confirmation of positive screening test reactors), a different set of priorities will come into play including high DSp, fast turn-around-times, technical sophistication and interpretative skills. There are a growing number of point-of-care or pen-side tests that have their own set of additional robustness and ruggedness requirements given the variable conditions of the environment in which they will be used and the skill level of the of operator who will performing and interpreting the test.

Aspects affecting choice of test

- Is the assay to be used for screening or confirmatory purposes, or both?
- Will the assay be used as a laboratory or field based test?
- Will it be used for one or more species? Which ones?
- Will the test be used for a group, sero- or strain-specific assay?
- Will the test be applied nationally or internationally?

The antigen ELISA is conceptually the same method employed for the antibody ELISA (Appendix 1.1.4.1), with the exception that antigen is the targeted analyte, and antibodies are the primary reagents used for capture and detection of antigens. Depending on whether the antigen is adsorbed directly on the microplate or captured by antibodies on a solid phase, along with subsequent detection steps, different formats are available.

It is not always apparent which assay format should be used to best fit the intended purpose. Availability of reagents and the limit of detection of the assay for a particular application may be significant factors in limiting the choice. Since many of the systems now target highly specific antigens, the choice of antibody for both trapping and/or detection becomes critical.

Preparation of the test sample is also a critical consideration depending on the test format being used. The use of trapping or capture antibody in sandwich-type assays enhances selectivity and reduces potential matrix effects. For assays requiring direct application of the analyte to the solid phase, preparatory extraction, centrifugation or filtration methods may be necessary to remove extraneous material. For a more in depth discussion of these different assay formats, please see Crowther (2001).

Of critical importance is the size and complexity of antigen and the availability of relevant reagents, such as capture antibodies (e.g. antigens to be detected in sandwich assays must have at least two unfettered epitopes) which limits this assay type to relatively large antigens or whole pathogens. The affinities of the immunological reagents come into play as the stability of the resulting antibody-antigen complexes in the microplate or on beads will affect the performance characteristics of the assay.

Practical concerns are availability and use of antigen standards for quality control and assurance purposes, repeatability, reproducibility, throughput capacity, turn-around-time of a test result, cost and technical sophistication and interpretation skills.

Working with exotic and/or zoonotic agents requires particular attention to biosafety and biosecurity regulations (see also Chapter 1.1.2. Biosafety and biosecurity in the veterinary microbiology laboratory)

c) Proof of concept experiments (feasibility studies)

The same types of experiments used in antibody detection tests are required for antigen detection tests (see Appendix 1.1.4.1).

d) Samples and data expression

i) Selection, storage and use of control samples for test development and validation studies

It is important to assess and monitor sensitivity and specificity of the test during development and validation. This is achieved by selecting several samples (4–5 is adequate) that range from negative to high levels of antigen of the analyte in question. These samples are used in experiments designed to optimise the assay. To achieve continuity of evidence requires care and forethought in preparation and storage of samples. A large volume (e.g. 10 ml) of each control sample is acquired and divided into 0.1 ml aliquots for storage at or below –80°C. One aliquot of each sample is thawed, used for experiments, and ideally then discarded. If it is impractical to discard the aliquot, it may be held at 4°C between experiments for up to about 2 weeks; however, there is a possibility of sample deterioration under these circumstances. Then, another aliquot is thawed for further experimentation. This method provides the same source of sample with the same number of freeze–thaw cycles for all experiments (repeated freezing and thawing of samples can denature antigen and/or facilitates the growth of other unwanted microorganisms and should be avoided). Also, variation is reduced when the experimenter uses the same source of sample for all experiments rather than switching among various samples between experiments. This approach has the added advantage of generating a data trail for the repeatedly run samples. After the initial stages of assay validation are completed, one or more of the samples can become reference reagent(s) that are the basis for data expression and repeatability assessments both within and between runs of the assay (Jacobson, 1998). They may also serve as in-house working standards if their activity has been predetermined; such standards provide assurance that runs of the assay are producing accurate data (Wright, 1998).

ii) Normalisation of results and their expression

The same normalisation procedures as used for antibody detection assays are applicable to antigen detection assays (see Appendix 1.1.4.1 for details).

e) Optimisation

The aim of this stage is to finalise test parameters related to reagents, consumables and equipment that will lead to a fixed protocol and will be used during the assay validation pathway Part B. For details, see Appendix 1.1.4.1.

f) Inhibitory factors in sample matrix

Due to a higher variety and complexity of specimens antigen detection assays are more likely to be influenced by matrix factors than antibody detection assays, which normally detect antibodies in serum. Inhibitory substances are frequently found in complex matrixes such as pus, semen, tracheal/nasal/cloacal swabs and may have an impact on the test result. Although ELISA antigen detection systems are rather resistant to inhibitory factors, please see Chapter 1.1.4/5., Section A.2.e, and Greiner *et al.* (1997) for descriptions of the type of inhibitors that could affect the assay. These references should be reviewed carefully to assure that all inhibitory factors are accounted for and controlled.

Optimisation and standardisation

- Is the test able to distinguish between samples with and without the analyte (what is the limit of detection = analytical sensitivity?)
- Does the test cross-react with non-target antigen in the sample or sample matrix (analytical specificity)?
- What is the noise or background activity in a negative sample?
- Has the repeatability been assessed for a range of control samples over a number of days
- Are sufficient positive and negative samples on hand to carry out the experiments for optimisation and validation
- If yes, are reference and control samples aliquoted and stored properly to avoid introduction of sample related bias (sample deterioration)?
- Have all critical reagents been run against each other in checker board titrations?
- Did you find optimal concentration/dilutions for each reagent?
- Did you include reference reagents and working standards/controls and did you normalise the OD data to achieve the best possible comparative results?

g) Calibration to reference sample and comparison to standard test method

Appendix 1.1.4.1 contains information relevant to this procedure.

B. ASSAY VALIDATION PATHWAY

1. Stage 1 – Analytical performance characteristics

a) Repeatability

See Appendix 1.1.4.1.

b) Analytical specificity (ASp)

Analytical specificity is defined as the degree to which the assay distinguishes between the target analyte and other components that may be detected in the sample matrix (see Appendix 1.1.4.7., Group B.a). The higher the analytical specificity, the lower the number of false positive results. Analytical specificity should be determined by testing well characterised samples from similar or related pathogens, which produce similar lesions as the target pathogen or are frequently found in samples containing the target pathogen. For example, to assess the analytical specificity of an FMD antigen detection ELISA for one particular serotype (e.g. serotype O), it is necessary to assess its reactivity of all sub-strains within this serotype (e.g. O Campos, O Manisa, etc.) to assess inclusivity. At the same time it is important to assess that the test does not cross-react with other serotypes such as A, Asia 1, C, SAT 1, 2 and 3 as a measure of exclusivity. Finally, there is also a need to assess whether the test cross-reacts with agents from diseases which may produce similar symptoms, e.g. vesicular stomatitis, swine vesicular disease and swine vesicular exanthema. Another example is an ELISA designed to detect avian influenza virus. As a screening test the assay should detect the nucleoprotein or matrix antigen of all subtypes, e.g. H1-H16 and N1-N9 (inclusivity). However it should not cross-react with viruses which cause similar clinical symptoms such as Newcastle disease or infectious bursal disease (diagnostic specificity) or with other non-specific components present in the matrix or on the solid phase (selectivity). Some ELISAs may be subject to false positive results attributable to non-specific factors, such as non-specific binding of antibody conjugates to the plastic surface and may require use of blocking agents. Care must be taken to eliminate these types of errors. For details on inclusivity, exclusivity and selectivity as sub-elements of ASp, see Chapter 1.1.4., Section B.2.b.

Analytical performance characteristics

- Has repeatability been established for a range of positive and negative samples within and between runs of the assay?
- Have upper and lower control limits of the assay been established?
- Have you defined ASe and ASp for this assay?
- Does the candidate assay compare favourably with a standard test method, based on objective quantitative and qualitative criteria?

c) Analytical sensitivity (ASe)

Analytical sensitivity is synonymous with the lower limit of detection (LOD) of antigen concentration in a sample. LODs are usually determined by endpoint dilution in which replicates (preferably 10) of each dilution in a $\log_2$ dilution series are run in the assay. The larger the number of replicates, the more precise the

determination of the dilution at which the antigen is no longer detectable. Accordingly, the last dilution in which at least 50% of its replicates are positive constitutes the LOD in the assay.

Screening assays or assays which are designed to detect sub-clinical infections or carriers should have a very high ASe. In these cases it may be difficult to obtain suitable samples and to determine the comparative ASe by running a panel of samples on the candidate assay and on another independent assay. If available, serial samples from experimentally infected animals could provide temporal information about the assay's capacity to detect antigen over the course of infection. For further details on ASe, see Chapter 1.1.4/5., Section B.2.c. and Appendix 1.1.4.5., Section 1.

d) Standard test method comparison with the candidate test method

See Appendix 1.1.4.1.

2. Stage 2 – Diagnostic performance characteristics

See Appendix 1.1.4.1.

a) The challenge in establishing accurate estimates of DSe and DSp for antibody assays

For all antigen detection assays including ELISAs, particular consideration must be given to the timing when the sample is taken as the probability of detecting antigen or the pathogen itself is very closely linked to the stage of infection. The diagnostic window will likely be much smaller in antigen/pathogen detection assays than in antibody detection tests as immune responses can normally be measured over an extended period of time. Dynamics of infection, e.g. acute, persistent, sub-acute, chronic or carrier-state are important determinants for sampling recommendations. For example, during an acute viral infection, the sample should be taken as early as possible after the onset of clinical signs. In persistent, sub-acute, chronic or carrier animals there is a balanced relation between the pathogen and the host and the agent may be present in minute concentrations which may be very difficult to detect. During the course of pathogenesis, other organ systems may become involved and different tissues or fluids may be more appropriate and/or beneficial for sampling as the disease progresses.

Diagnostic performance characteristics

- Are the criteria used to determine the positive and negative reference populations legitimate?
- Do the reference samples fully represent the population targeted by the assay?
- Were there difficulties in obtaining a sufficient number of samples? If so, how was the problem addressed?

b) Reference animal populations

i) Animals of "known infection status"

Depending on the composition of positive and negative reference samples, the same test may have different estimates for DSe and DSp. Ideally the composition of reference samples and animals should match as closely as possible the samples which are expected from the target population for which the test was developed. It is important to have a clear 'case definition'. This is a set of criteria used to decide whether an individual or group of animals is infected or not. The reference status must relate to the purpose of the testing. For example, if the purpose of the assay is to be used as a screening test to detect early infection of FMD (e.g. in free-ranging cattle with vesicles), then the majority of samples to determine DSe and DSp should the taken from this target population. Relevant information should be collected and summarised for all animals involved at this stage of validation (e.g. species, age sex, breed), and information on other factors that are known to influence DSe and DSp (e.g. date and place of sampling, immunological status, vaccination and disease history, pathognomonic and surrogate tests used to define status of animals, prevalence within population and description how the reference status was derived).

A sample from a negative reference animal refers to lack of exposure to or infection with the agent in question. For example a brucellosis negative population could be defined as a region with cattle herds, without clinical cases of brucellosis in recent years supported by negative serological tests such as Rose Bengal, ELISA and CFT and negative microbiological testing of aborted fetuses and placenta. Samples from these animals fulfill the status of negative reference samples.

If vaccination is carried out, it may interfere with antigen detection (e.g. vaccination with live *Brucella* S19 vaccine). Samples from these animals should not qualify as negative reference samples.

The types and limitations of reference standards commonly used for evaluation of the performance characteristics of a new assay are listed here. An expanded description each reference standard is provided in Appendix 1.1.4.1., Section B.2.b.i. and in Jacobson, 1998. The strengths and limitations of these reference standards must be considered carefully when using samples, derived from animals

that fall into any of the following four categories, as sources for establishing DSe and DSp for a candidate assay (Jacobson, 1998).

- ***An unequivocal reference standard*:** presence of the agent in the host or evidence of definitive histopathology.
- ***A composite reference standard:*** verification of uninfected or unexposed animals.
- ***A relative reference standard*:** comparative serology (see Appendix 1.1.4.6. for an example drawn from PCR but applicable to antibody detection systems).
- ***An adjunct reference standard*:** experimental infection or vaccination (see Chapter 1.1.4/5., Section 3.c. for significant limitations of this type of standard). Note: In the context of antigen detection, "comparative" tests should include pathogen detection by isolation, culture, NAD tests and/or histopathology or other *in situ* techniques.

ii) Latent-class models for sample selection

For a discussion of this approach to sample selection, see Chapter 1.1.4/5., Section 3.b.ii and Statistical approaches to test validation Appendix 1.1.4.5.

c) Threshold (cut-off) determination

It is important to clearly describe the method and the samples used for selecting a cut-off. It is strongly recommended to conduct an ROC analysis to show the potential performance of the test in other epidemiological settings. The procedures for establishing the cut-off between negative and positive results of antibody assays are as described in Chapter 1.1.4/5., Section 3.d.

3. Stage 3 – Reproducibility and augmented repeatability estimates

Reproducibility assessments for antigen detection assays are not uniquely different from similar assessments for any other type of assay. Therefore the reader is directed to Chapter 1.1.4/5., Section 4, for details on reproducibility analysis and for reference samples and panels to Appendix 1.1.4.7. The OIE Quality Standard (World Organisation for Animal Health [OIE], 2008) provides guidelines for laboratory proficiency testing.

4. Stage 4 – Programme implementation

a) Interpretation of results

See Appendix 1.1.4.1.

5. Monitoring assay performance

a) Monitoring the assay

After performance characteristics of the new test have been established, ongoing monitoring, maintenance and enhancement are required. It is important to keep on monitoring the repeatability and reproducibility of the assay over time, e.g. quality control samples have to fall within pre-established limits. If not, the test is not valid and has to be repeated. This is an important way to detect changes or trends in the assay. Simple analysis of results, e.g. statistical assessment of mean values, standard deviation and coefficient of variation are useful in this process and results can be plotted in a control chart. Participation in external quality control or proficiency testing programs is useful to identify random and systematic errors and provide credibility in test results. Further information about this topic can be obtained from Crowther *et al.* (2006) and in Appendix 1.1.4.1.

b) Minor modifications of the assay - replacement of depleted reagents

Over time changes in the test protocol may be necessary due to better or cheaper reagents or because the target analyte has changed. Batch-to-batch variation of biological reagents is considered a major contributor to test variation. When reagents such as antibodies or antigen must be replaced they should be produced or procured using the same protocols or criteria as used for the original reagents. New biological reagents (e.g. control samples, antigen, capture or detection antibodies, conjugate, chemicals or consumables) need to be assessed for comparability. Appendix 1.1.4.5. gives an overview acceptable comparability studies. A ground rule is never to change more than one reagent at a time in order to avoid the compound problem of evaluating more than one variable concurrently (see also Appendix 1.1.4.1).

c) Major modifications of the assay – changing to a new ELISA type

It is a major challenge of laboratory diagnosis to keep up with the evolving nature of infectious pathogens. Over time pathogens may change their antigenic characteristics and new strains may emerge, e.g. the new Bluetongue virus strain in 2009. This requires a full test development and validation study. Another major change is the use of a test in a different species that it has been originally validated for, e.g. it may be necessary that an FMD Ag detection ELISA, which has been validated in cattle has to be used in camelids or buffalo in different geographical regions, e.g. from a European country into a sub-Saharan environment. Evaluation of reference samples that represent those populations of Stage 2 in Fig 1 of Chapter 1.1.4/5. will accomplish this requirement (see also Appendix 1.1.4.1).

d) Enhancing confidence in validation criteria.

Due to the extensive set of variables that have an impact on the performance of antigen detection assays, it is useful to expand the number of reference samples when possible, due to the principle that error is reduced with increasing sample size (See also Appendix 1.1.4.1.).

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ADVICE FOR MEMBER COMMENTS

Appendix Number and Title: 1.1.4.3. Development and optimisation of nucleic acid detection assays

Country making the comments

Date

NB: The Biological Standards Commission is particularly interested in OIE Member Country opinion on this appendix (and its accompanying chapter and six other appendices): are the documents clear and useful? If not, how they could be improved?

It would be appreciated if the following guidance notes are followed in making a reply:

1. *Comments may be general or specific, but specific comments are usually more valuable. General comments should be such that some conclusion and action can be taken in response to them. For example, it is of little help to make statements such as "This test is no longer used in our laboratory", without indicating the reasons and what test is used instead.*

2. *Specific comments should be identified, by indicating the line number in the text, to facilitate the making of editorial changes.*

3. *The pointing out of typing or technical errors is welcome, but the correct word or figure should be indicated. For example, it is of little help to say that 0.8 M is too high, if the preferred value is not indicated.*

4. *Bear in mind that the introductory chapters are not intended to be exhaustive, and indeed none of the chapters can give a completely comprehensive cover of the subject, otherwise the* Terrestrial Manual *would be too long. However, assistance in indicating priorities is always helpful.*

5. *The* Terrestrial Manual *is intended for world-wide use. The chapters need to reflect the development of new technology, but the old established methods, usually requiring less sophisticated apparatus, have to be retained. New technology should not be described in detail until it has gained wide acceptance as a reliable method.*

General Comments

Specific Comments (*add continuation sheets if required*)
line:

APPENDIX 1.1.4.3.

DEVELOPMENT AND OPTIMISATION OF NUCLEIC ACID DETECTION ASSAYS

INTRODUCTION

An ever increasing number of nucleic acid detection (*NAD*) *tests are now being used for diagnosis of infectious diseases in various species of animals and man. The most common methods are the polymerase chain reaction* (*PCR*), *of which there are a number of variations and isothermal amplification methods* (*such as loop mediated isothermal amplification*). *In addition, solid-phase or liquid phase microarrays are increasingly appearing as new tools of biotechnology-based diagnosis. The amplification techniques employed in these assays make them highly analytically sensitive. The product of the amplification reaction can be detected in a number of ways, for example, by visualisation in agarose gels, using labelled probes* (*e.g. TaqMan probes*) *to detect accumulation of product in real time or by using arrays where specific probes are captured on a solid-surface matrix or beads* (*Lauerman, 2004; Viljoen* et al., *2005*). *Different PCR assays can be multiplexed together to detect several targets in one tube or to combine targeted analytes and controls that generate different amplification products, all in one reaction vessel. Whilst this has obvious advantages, great care must be taken during optimisation to ensure that assay performance is not compromised. Similarly a multiple outcome PCR can be created using one set of primers, but employing tagged probes, which bind to different target sequences in the different species or strains detected by the PCR* (*Wakeley* et al., *2005; 2006*).

NAD amplification techniques are usually based upon the principle that there is an exponential amplification of the specific sequence targeted in the reaction. This makes the assay highly analytically sensitive but can, under some circumstances, result in contamination of other samples with the products of previous reactions. This is more likely to be a problem where reaction tubes are opened in the laboratory for further processing, for example, to run gels or to perform nested assays. To avoid such contamination, strict laboratory protocols should be employed involving separate rooms or cabinets for particular stages of the assays, changes of laboratory gowns and gloves, and stringent cleaning programmes (*Viljoen* et al., *2005; Subcommittee of Animal Health Laboratory Standards*). *For these reasons, tests based on closed tube systems are generally more suitable for diagnostic assays* (*Sawyer* et al., *2006*). *As with all assays, it is important to use appropriate controls to prove that the assay is performing as expected. All samples used in assay development should have well established provenance and the development should be carried out within the framework of a quality system to ensure appropriate levels of training, equipment maintenance and monitoring etc.* (*Burkhardt, 2000*).

A. ASSAY DEVELOPMENT PATHWAY

1. Definition of the intended purpose(s) for an assay

Purpose of the assay:

- Is it for a screening or confirmatory test, or both?
- Is it for detection of a group of pathogens?
- Is it for detecting a single virus or strain?
- Is it for discriminating between vaccinated *and infected animals*?

The first consideration in assay development is to clearly define the specific purpose and application of the test to be developed and to understand how it will be applied because this informs many of the decisions of the development pathway. For example one might choose to develop a screening test to detect all avian influenza (AI) strains in birds for which an inclusive and broadly reactive test is necessary, or to determine the haemagglutinin type, in which case a more specific test is needed. For some tests the requirement is to detect a group of viruses, e.g. the pan peste virus PCR that detects bovine viral diarrhoea (BVD), border disease and classical swine fever (CSF) viruses. For other tests, the

requirement may be to detect a single virus, or sometimes even to allow a DIVA approach. An example of such methods are the recently published real-time RT PCR assays for CSF virus which were developed for the genetic differentiation of naturally infected from vaccinated wild boar (Liu *et al.,* 2009).

2. Assay development – experimentation

a) Quality assurance

It is important that assays are developed in laboratories where high standards of quality assurance and control are employed (see *Terrestrial Manual* Chapter 1.1.3.). The validation data for test performance and accuracy determined during the development and validation phase must be robust as it will form the basis for interpretation of disease status and consequent actions when the assay is routinely used.

b) Reference materials

Sample selection (see *Terrestrial Manual* Chapter 1.1.1. Collection and shipment of diagnostic specimens)

For most diseases, the samples required for NAD assays are likely to be similar to those used for current detection methods such as bacterial or virus isolation. For example, detection of AI by real time RT PCR and virus isolation use the same samples. Recently, swabbing of fresh cuts in organ samples proved to be a practical approach, replacing the laborious homogenisation of organ samples. Similarly, there is a trend to use samples, which can be obtained by non-invasive methods, such as saliva for detection of PRRS in pigs (Prickett *et al.,* 2008) or bulk milk samples for determination of herd BVD status. Prior to the selection of pooled samples, such as bulk milk, test developers need to consider and assess the implications of dilution of the target analyte on diagnostic sensitivity and incorporate this into the validation plan (see below).

- What samples will be used from the target population?
- What are predilection sites for the agent in the host?
- What sample collection, storage and transport method is anticipated and what are the possible effects on results?
- Will samples be single or pooled?
- Will samples in the validation panel be representative of the target population

It is important to understand the biology of the pathogen concerned and the nature of the sample collection devices. As in the case of AI, different strains of organisms have different predilection sites in host birds. For example cloacal samples are appropriate for some strains, whereas buccal samples are acceptable for others. Therefore, a test for use in a surveillance programme would need to incorporate both sampling sites and be validated using both types of sample. Another significant factor is the matrix in which the analyte resides in the host. Cloacal samples are more likely to contain PCR inhibitors than buccal samples. Another potential confounding factor is the type of swab used to collect the samples; some contain materials that inhibit PCR reactions. Therefore, it is very important to precisely specify the preferred sample material and to fully describe the swabs and swabbing protocol, including the preferred buffers or transport media and storage conditions.

Consideration also should be given to whether samples should be tested singly or in pools and whether pools should be of different samples from a single or multiple animals. Any pooling strategy should be precisely defined and validated prior to use. Finally, if the target population is “birds”, the validation study should cover a large representative population of different species to demonstrate that the assay is widely applicable, whilst concentrating on the most prevalent species or those used as sentinels of infection.

c) Design of test method

i) Choice of test

For most surveillance activities large numbers of samples may be tested first by a screening assay. In the above example for AI, the screening assay described must detect all known strains of Influenza A, either in a particular region or throughout the world. The test must be highly sensitive so it does not miss true positive samples, and analytically specific (inclusive) for detection of all viruses in the Influenza A group.

Avian Influenza is a high profile disease. If a new test should generate a high proportion of false positive results that cannot otherwise be confirmed, the infection status of the birds would be difficult to

- Is the test for use only in a particular region or world-wide?
- Is it sufficiently sensitive and is the analytical target inclusive enough to not miss positive samples?
- Consider the impact of a high proportion of false positive results that cannot be confirmed then what?
- Are rapid results possible and/or necessary?
- Will confirmatory tests (an analytical tool) be used to determine which strain?
- Will determination of the strain have important bearing on the action taken?

resolve. Exclusion of closely related agents that are not of interest within context of the intended purpose of the assay is essential.

It is also important for the test to produce rapid results so that disease control measures can be swiftly applied. The logical choice of tests would be a real time PCR using TaqMan probes or a field based assay. For a TaqMan assay, primers and probes are likely to be based upon the Matrix (M) gene which is known to be present in all influenza type A isolates. It might be important to develop confirmatory methods for use in conjunction with the screening test to determine which particular strain of AI is present and whether the strain is highly pathogenic, because this has an important bearing on the control measures that may follow. Such confirmatory methods, that further characterise samples, would be classed as analytical tools and only are applied to the subset of samples that test positive using the screening assay.

ii) Method design

The method should be carefully designed to fulfil the purpose that was defined at the outset. Important factors to consider will include application, types, and numbers of samples to be tested. This, in turn, leads to logistical and practical considerations for the candidate assay, such as whether it will be conducted in a laboratory, with or without automation to achieve high throughput, or in the field using a pen-side assay. All available information such as publications, sequences deposited in databases and in house sequence data should be used. Sophisticated software is available to optimise primer and probe design; computer modelling of probable sequences for use in the assay is a first step.

Has the design been shaped by the intended purpose of the assay?
- What is the specific application?
- What are the types and statistically relevant numbers of samples to be tested? (See Appendix 1.1.4.5.).
- Is automation required?
- Should the test be field or lab based?
- Has exhaustive evaluation been done on publications of sequences?

d) Feasibility study

Before embarking on the validation of a newly developed assay, a feasibility study should be carried out using a small panel of approximately six or eight well-characterised samples to assess whether the system is viable. The panel should consist of samples distributed across the operating range of the assay, i.e. at least two negatives, at least two unambiguously positives, and ideally samples falling mid-range. Ideally these samples should come from different animals. The assay should achieve as wide-as-possible separation of test results for the high positive and negative samples in this panel. It may be prescient to test a dilution series (analyte diluted in matrix) at this point to assess relative analytical sensitivity if the test is a potential replacement for another method where sensitivity is an important criterion (See Appendix 1.1.4.6 Comparability of assays after minor changes in a validated test method).

- Was the assay first evaluated on a small sample panel (six to eight samples) to assess viability of the approach?
- Was there good separation between test results of negative and positive samples?
- Was a preliminary test on a dilution series in matrix to preliminarily assess relative analytical sensitivity made (if that is an important criterion)?

e) Development & optimisation

The aim of this stage is to define and optimise the method that will be used to carry out the test in future routine testing. This includes description of appropriate facilities to carry out the assay. It is important to consider both the method of sample extraction required to prepare samples for the test and the assay procedure. For any PCR assay, definition of clean room protocols is required to minimise contamination. If large numbers of samples are anticipated, selection of an automated extraction procedure may be essential (Jungkind, 2001).

- Optimal location? Use a clean room or cabinet system to minimise contamination
- Optimal method of sample extraction to prepare samples? Done manually or by automation?

Usually, it is necessary to assess a number of different test methodologies and vary concentrations of reagents, template additions and reaction times to optimise the extraction and the assay. It is important to either change only one variable at a time or use a multi-factorial design and analysis (see Chapter 1.1.4/5., Section A.2.f on Robustness). It is important to identify which factors have only a narrow range of optimal activity, as these are critical points in the assay procedure and may affect an assay's robustness. Generally, the assay stages are relatively simple to optimise, but care

- Have concentrations of reagents been tested for optimal reactivity?
- Has extraction been optimised?
- What are the template additions and reaction times to optimise the assay?
- Which factors have a narrow range in which they perform optimally?
  - Have you defined that range?

needs to be taken to ensure that a robust extraction method has been developed which is suitable for routine laboratory application (Burkhardt, 2000).

Controls for PCR are many and varied. It is important to include appropriate controls to show that the assay is performing as expected. The various controls that need to be considered include:

i) A host-species control

This control demonstrates that the swab had been in contact with a target species and that the sample contained "bird" nucleic acid, which is available for extraction using the defined protocol. The most suitable target for this control is a housekeeping gene such as beta actin, which is present in the target host species. This is relatively simple if the host species is a single species e.g. chickens, but it will be more challenging to find a suitable target for all "bird" samples. This type of control may not be required if a sample is added directly to the extraction process, such as a piece of tissue or blood, but is recommended for "indirect" samples such as those collected on swabs; in this case, a the host-species control should be used for every sample tested.

ii) No-template control

This control reveals whether contamination of the sample has occurred, resulting in amplified product when no amplified product should be present, as in a sample containing no targeted sequence. Consideration should be given to the number and placement of no template controls in the assay set up template. Generally, a number (approximately 5% of wells) of no-template controls are distributed randomly over the plate.

Have you included all necessary controls to prove the assay is performing as expected?
- Template control?
- Inhibition control?
- Positive sample control?
- For RNA assay – reverse transcription control?

iii) Analyte-positive control

This positive control, having Ct activity within the defined operating range of the assay, is used on each plate. The most suitable choice for this control may be a plasmid containing the target sequence, which can be used to check for the expected level of amplification in the assay. However, this does not assess the efficacy of the extraction process, which requires a known field sample or its equivalent.

iv) Inhibition control

This control is needed to detect possible inhibitors of the PCR reaction. If an inhibition control yields a negative result, this infers that the sample contains inhibitory substances. and that a negative test result for the test sample cannot be interpreted as "negative" because of likely inhibition. Certain samples such as faeces and semen often contain inhibitors whereas this is less of a problem when testing blood samples or cultured organisms (Ballagil-Pordány & Belák, 1996). Data collected during the validation process will allow for a risk-based decision as to whether an inhibition control should be included for each sample or the test system is unlikely to be affected by inhibition. If inhibitory substances are a significant problem an inhibition control must be included for each test sample.

- Did you consider all of the possible pitfalls in use and interpretation of results for this inhibition control?
- Is the purpose and application of the inhibition control clearly specified in the assay protocol?

There is considerable debate about what to use as the most suitable and effective inhibition control. Generally this could be one of the following:

- An artificial target, such as a length of DNA contained in a plasmid, which is added to the extracted sample and amplified with the same primers as the test target, but is of a different size or contains a different internal sequence so that it is identified as the internal control when the detection method is applied. The advantage of this approach is that it utilises the same primers employed in the test, and the control can be added at precise concentrations. However care must be taken during assay optimisation, so that the analytical sensitivity of the assay is not detrimentally affected because of competition for primers. The other disadvantage is that as an added component, it only controls the assay stage of the test and does not act as a control for the extraction stage.

- An alternative strategy is to amplify a housekeeping or structural gene such as β-actin which always is present in the target tissue, and thus the sample. If the housekeeping gene has been inhibited by substances in the sample, the inference is that amplification of the gene targeted by the test may also be inhibited. This conclusion is not always warranted. Housekeeping genes are often present in abundance, so sometimes can be detected even in the presence of inhibitory substances. But the more limited amounts of the assay's targeted sequences may be inhibited

from amplification. In this case, the amplified and detected housekeeping gene was not a sufficient control for inhibitors, resulting in a false negative inference for the test result. However, if properly used, an inhibition control, which is naturally present in the sample controls can be a useful control for inhibitors for the whole assay including sampling, storage and extraction.

- For assays based on RNA targets, a control can be included to assure that reverse transcription of RNA to cDNA has proceeded appropriately. This control should be included during the validation process to assess whether reverse transcription is likely to be an issue. A risk-based decision can be made following analysis of the validation data as to whether the control should be included routinely when the assay is run.

f) Inhibitory factors in the sample matrix

Generally for NAD methods, pure cultures, blood and most tissues are relatively easy to work with and extraction of amplifiable NA is generally successful. Faecal samples, semen and autolysed tissue can be more challenging because they generally contain more inhibitors. It is vital to have a robust and repeatable sample extraction procedure, which is appropriate for the numbers of samples to be handled (automated if necessary) and to utilise inhibition controls if necessary (see section above).

g) Operating range of the assay

The operating range of the assay should be determined by diluting out a high positive sample and plotting the range of results obtained versus known amounts of nucleic acid (concentration, dilution, number of genomic copies, etc.). This reference sample must be in the same matrix as the test sample, i.e. it is not appropriate to determine the operating range of a sample diluted out in buffer, if the usual matrix is blood.

- For determining the operating range of the assay, did you dilute the samples in matrix of samples for which the test is intended?
- Does the operating range of the assay conform with the expected norms for such an assay?

h) Robustness

An assay should tolerate small changes in concentrations of reagents and/or slight variations in processing times and temperatures used for different stages of the assay. This can be determined during assay optimisation when critical stages are identified. Such stages that tend to be variable should be well described in the assay protocol so that particularly exacting processes are assured for carrying them out. This is a laborious process that ultimately is monitored for precision and accuracy by internal and external quality control samples run in the assay.

- Does the assay tolerate small changes in reagent concentration or in physical parameters?

i) Calibration of the assay to reference samples

Ideally, international or national reference standards should be used to calibrate the assay. However, these are not always available so it may be necessary to create an in-house reference standard. A working standard(s), for inclusion in all runs of the assay, needs to be created, aliquotted, and stored in sufficient quantities for use in every run of the validation process and for routine use after the validation has been completed. The working standard(s) could be multiple aliquots of a particular sample, which can be used within each assay run. They could also consist of a plasmid containing the sequence of interest, spiked into sample matrix. Use of the latter allows the test developer to determine the number of genome copies that can be detected by the assay. In some instances test sample results are “normalised” by comparison to the working standard sample(s) included in each run of the assay. This allows direct comparison of data between runs (Huggett *et al.,* 2005; and Chapter 1.1.4/5.).

- Have you calibrated the assay to external reference standards?
- If external reference standards not available, have you created an in-house reference standard?
- Have you made working reference standards in sufficient amounts for use in all development and validation experiments?

B. ASSAY VALIDATION PATHWAY

Once the protocol for the assay has been developed and optimised, it must be fixed and held constant while being evaluated through the stages of the validation pathway.

1. Stage 1 – Analytical performance criteria

a) Repeatability

- Has intra- and inter-assay repeatability been determined?
- Is repeatability within the accepted range of coefficient of variation (CV) limits of 15% to 20%?

Repeatability of the assay is a measure of agreement between results (within and between runs) using the same test method in one laboratory. Usually a small panel of three (preferably five) samples covering the operating range of the assay is selected and tested using the entire assay procedure (including nucleic acid extraction). Within assay (intra-assay) variation is determined using multiple (at least five) replicates of each sample in this panel in one assay run. Between run (inter-assay) variations are determined by testing these samples over several days, using several operators and at least 20 runs. The repeatability panel should be tested treating all samples and each of their replicates exactly like individual diagnostic samples, subjected to every step from sample preparation to data analysis. Accordingly, every replicate of every sample is subjected to an independent extraction. This allows for determination of repeatability, both with and between runs of the assay that mimic future runs of the assay when implemented for diagnostic use. Minimal variation in repeatability is important, particularly near the cut-off(s) that establish positive, inconclusive and negative ranges, because higher variability can result in incorrect interpretations (see Appendix 1.1.4.4. Measurement uncertainty).

For real-time PCR, a variation of 1–2 Ct values for a single retested sample is considered acceptable. Repeatability can also be expressed as a coefficient of variation; in this case a CV of 5–10% is acceptable. The assay should be designed so that the decision point (cut-off) lies on the steepest part of the real-time PCR Ct curve. If this is achieved, repeatability will be optimal at the critical point of the assay. (Larger CVs at the clearly negative and highly positive ends of the operating range of the assay do occur and have little impact on test result interpretation.)

b) Analytical specificity (ASp)

Depending upon the intended purpose of an assay, its analytical specificity is determined by the selected genetic sequence(s) of an organism(s) targeted by the assay. The assay can be designed to be highly selective, with analytically specificity for a single genetic sequence that is not known to be present in other organisms or strains of the targeted organism. Such an assay is said to exhibit exclusivity and connotes a confirmatory assay of high ASp.

Alternatively, the assay may be designed to target a conserved genetic sequence that is common to several strains of a given species, or several species of a genus. Such an assay has an ASp that exhibits inclusivity, making it useful as a screening test. For an inclusive screening assay, the analytical specificity should be determined by testing all lineages, strains, species, etc., that the assay is expected to detect. The assay should then be evaluated for its capacity to exclude related organisms such as non-pathogenic strains that are not of interest to the intended purpose of the candidate assay.

- Has a panel of as many well-characterised isolates of the target pathogen as possible, including isolates from a variety of geographical areas and hosts, been tested?
- Have related organisms and pathogens, which cause similar clinical syndromes, been tested?

In the example of an AI screening test, the assay should be run against as many well-characterised isolates of AI virus as are available to assure that all strains from a variety of geographical areas and hosts are detected (i.e. to demonstrate inclusivity). This is generally done using laboratory strains/cultures or nucleic acid. For AI, different lineages of the virus exist such as the Asian, European and North American strains. It is important to consider how the assay will be used and in which geographical regions. That will aid in determining whether it is necessary to evaluate some or all of these lineages. Another important factor is that viruses can change rapidly and mutations can render a test sub-optimal. An example of this is the appearance of the recent pandemic strain of H1N1. The analytical sensitivity of the PCR for the traditional M gene was impaired for this strain because the sequence that rendered the assay specific had mutated. In most countries new primers have been introduced and used either as a new test or in combination with the traditional M gene primers as a combination test.

The discriminatory power of the assay should be checked by testing organisms related to the AI virus. These would include pathogens which cause similar clinical syndromes such as Newcastle disease, infectious

bursal disease etc., and other organisms, which are likely to be found in the target sample (i.e. to demonstrate exclusivity).

c) Analytical sensitivity (ASe)

- What is the detection limit of the assay?
- How does the limit of detection compare to current standard methods?
- Is the ASe sufficient that it will be fit for its intended purpose?

There are two common approaches to assessing analytical sensitivity, also known as the limit of detection. The first is to use a dilution series of the target pathogen (in this case AI virus) diluted in sample matrix and not buffer. The dilution series is usually tested using the new assay under validation and a standard method. For AI, the standard method could be virus isolation or another in-house standard method of detection. This approach yields a comparative measure of the two methods. The second approach is to use a plasmid construct, containing the target sequence and test this as a dilution series in sample matrix. In this manner, the number of genome copies detectable by the test method can be estimated.

d) Standard test method for comparison with the candidate test method (see Appendix 1.1.4.6. Comparability of assays after minor changes in a validated test method)

- Has the new test performed in a satisfactory manner compared to a standard method of comparison?
- Is preliminary reproducibility data acceptable
- Does the assay merit proceeding to studies on full validation (Stages 2–4)?

On some occasions it is not possible to complete a full validation exercise either because samples of known analyte status are scarce (e.g. exotic diseases) or, when an emergency situation arises, the assay is required for use before it can be fully validated. Provisional recognition can be achieved provided that results through Stage 1 of the validation process compare favourably with results of a standard test method or a known established and preferably published method. Another choice for a standard method is the one used routinely in the laboratory. It is important to recognise that different methods identify different morphological or functional entities of the organism. It is therefore possible that comparison between a standard culture-based method and a new NAD technique will give rise to discrepant results (see Section B.2, Stage 2, below, for discussion on resolution of discrepancies). The choice of assessment panel is also very important and it should be as extensive as possible (see Appendix 1.1.4.7. Selection and use of reference samples and panels). If only a small panel of samples is available for evaluation, it is useful to determine if the assay withstands the rigors of use in other laboratories. This requires that both laboratories use the same protocol, and same reagents, same panel of samples and similar (if not identical) equipment.

If lack of samples prevents continuing through the next stage of the validation pathway (Diagnostic Performance of the assay), it is acceptable to use a NAD assay that has been provisionally accepted by having been thoroughly validated through Stage 1 of validation (see Chapter 1.1.4/5., Section B.2.g). Acceptance of provisional validation is fully dependent upon approval by local authorities, or through bilateral agreements between countries.

e) Analytical accuracy

Analytical tools, designed to provide information to characterise samples that are detected using a screening assay, only require validation of their analytical performance characteristics. So, there is no requirement to determine diagnostic sensitivity or diagnostic specificity in such cases. Examples of such approaches include PCR typing assays to determine whether a matrix-positive influenza A strain is H5 or H7, or methods to determine antibiotic resistance which are only applied to cultured bacteria. A nucleic acid based technology employed for such tests includes nano-array based methods, which introduce their own challenges due to the large amount of data generated for each sample tested. Before implementing such assays, consideration should be given to how this analysis can be accomplished. A simpler method approach is to compare the results of a new analytical tool to a standard tool and permit its use as long as the results of the new and existing technique compare favourably (Anjum *et al.,* 2007; Batchelor *et al.,* 2008; and Appendix 1.1.4.6.).

2. Stage 2 – Diagnostic performance criteria

Diagnostic sensitivity and diagnostic specificity

Diagnostic sensitivity (DSe) and specificity (DSp) are the primary performance indicators of a diagnostic assay. When determining these estimates it is vital to select sufficient numbers of samples which are relevant to the target population for the test under assessment). It can be difficult to obtain large numbers of samples (particularly positive samples) for some exotic diseases, which are not commonly found. In such cases, with few samples

available, the amount of error allowed in estimates of DSe and DSp, of necessity, may be rather large (see Chapter 1.1.4/5., Section B.3, Table 1).

Negative reference samples are often selected from animals living in regions where the disease is not present, while positive samples are usually obtained from animals with clinical signs which have been confirmed in the laboratory. This can lead to overly optimistic estimates of DSe and DSp because the samples do not represent the whole spectrum of the disease process, ranging from non-clinical animals which may have pathogen loads that are much different than animals experiencing fulminant or chronic disease.

Samples are often categorised using current test methods such as virus isolation (VI) or bacterial culture. However, this can be problematic when validating new molecular tests, because the basis of the two test systems is different. For example, a positive bacterial culture is dependent upon the presence of a viable organism whereas nucleic acid based methods detect genomic sequences of both live and dead organisms as long as the nucleic acid is still present in the sample. VI methods can be particularly susceptible to inhibitors and contaminants present in the sample matrix, leading to an underestimate of "true positives". This can result in apparent discrepancies where samples are positive using the new molecular test and negative by traditional methods. Various strategies for resolving such anomalies include, but are not limited to, sequencing (which can demonstrate that the pathogen of interest was present in a particular sample), or testing using another molecular approach.

An alternate method is available if samples from animals of known infection status are difficult to obtain. If an appropriate sampling design can be employed and different independent test methods used to test the samples, it is possible to obtain estimates of DSe and DSp by using Bayesian methods (latent class models), under certain circumstances. This has been particularly successful for resolving the lack of samples of known infection status (see Appendix 1.1.4.5.).

To calculate DSe and DSp estimates of the candidate assay, the test results first must be reduced to categorical (positive, negative, or indeterminate) status. This is accomplished by insertion of one or two cut-off points (threshold or decision limits) on the continuous scale of test results. For example, in some circumstances it is appropriate to use a cut-off for a real time PCR assay in the region of 35 Ct, which means that some samples that produce a higher Ct value are categorised as negative or inconclusive. For a different PCR assay, however, any sample which merely registers a Ct may be categorised as positive. The performance of a particular real-time PCR, comparative validation data, the ultimate application of the results generated, and any relevant veterinary information should be taken into account when considering the use of a cut-off.

- **Threshold and cut-off** are considered to be synonymous. A cut-off is the test value selected for distinguishing between negative and positive results on a continuous scale of test values.
- Indeterminate, intermediate, suspicious, borderline, grey zone or equivocal **are terms used synonymously for a zone of test values falling between the positive and negative cut-offs.**

3. Stage 3 – Reproducibility and augmented repeatability estimates

Reproducibility is a measure of the agreement between results obtained in different laboratories using the same protocol, similar (preferably the same) equipment and the same panel of samples. Ideally the panel would consist of 20–30 samples including a few which are present as quadruplicates. The panel should consist of samples that cover the dynamic range of the test with several that have activity close to, and on either side of, the test-cut-off(s). The same panel used for determination of repeatability could be used for this evaluation, but with enhanced numbers of replicates. Measurements of precision can be estimated for both the repeatability and reproducibility data (see Appendix 1.1.4.4. Measurement uncertainty for further explanation of this topic and its application). Appendix 1.1.4.5. provides further information about the selection and use of reference panels.

4. Stage 4 – Programme implementation

a) Interpretation of test results

Best practices for programme implementation are general to all assay types (see Chapter 1.1.4/5.). For PCRs, an inherent advantage is the possibility of follow-up genomic sequencing to resolve apparent false positive results. Assays including PCRs are often validated using similar numbers of positive and negative samples. However, in surveillance programs, they are often applied to affirm the absence of the disease in question in locales where disease prevalence is very low and often approaching zero. In such circumstances, false positive results can be a significant problem even if the diagnostic specificity of a particular assay is high. If the DSp of an assay is 99.5% this means that one in 200 test-positive results will be false if the prevalence is close to zero. If a large number of samples are tested from a population of zero or very low prevalence, such false positive results can significantly out-number true positive results (see Chapter 1.1.4/5., Section B.5.a, Stage 4 of for further explanation of predictive values of test results as a function of

disease prevalence). For NAD assays used in such circumstances, it would be good practise to confirm PCR-positive results by sequencing.

5. Monitoring of assay performance after initial validation

a) Monitoring the assay

Monitoring of repeatability by charting the values obtained for working standard control samples provides re-assurance that the assay is performing as expected. Similarly, participation in proficiency testing schemes issued by external providers of quality assurance samples provides evidence of on-going reproducibility and also allows comparison of test accuracy if a reference standard(s) is included in each run for "normalisation" of data. Re-testing of a proportion (usually in the range of 1–5% depending on throughput) of retained samples is also employed by some laboratories to demonstrate that the assay is performing consistently between runs.

b) Minor modification of an existing validated assay

In time it may be necessary to modify the assay because the target analyte has changed, e.g. if the assay for avian influenza is to be applied in another part of the world or if new strains or lineages of a virus have emerged (see section B.1.b, above, on the evolution of the new pandemic lineage of H1N1). RNA viruses evolve rapidly and point mutations can occur, so it is advisable to regularly confirm the nucleotide sequences of the primer and probe sites to ensure that they remain appropriate.

i) Technical modifications

Modifications of an assay are likely to be required, over time. For example, use of different equipment, use of a different extraction protocol or automation of particular stages will minimally require comparison of the original validated assay with the modified version (see Appendix 1.1.4.6.). If results of the modified version fall outside of the operating range of the original assay, a revalidation may be necessary.

ii) Replacement of depleted reagents

It is important to assign unique identification numbers to all batches of reagents and to record the components used for particular assays. The most critical components in NAD assays are probes and primers. Current and new batches should be tested in parallel prior to their introduction. However, for other reagents it is usually sufficient to monitor batches to inform troubleshooting, should that become a necessity.

c) Major change in assay requiring revalidation

Upon occasion, application of the assay may need to be extended beyond the scope of the original intended purpose of the assay. Examples are inclusion of another host species or a population of animals from a different geographical area. In such cases it is important to revalidate the assay because of new biological considerations with their many associated variables. The precise details will depend on the extent of the change. Moving the assay into a new geographical area might mean that the analytical characteristics of the assay are still valid but that the diagnostic criteria need to be re-defined. Similarly modifications may be made to primer or probe sequences to allow detection of new strains. It will then be necessary to demonstrate how the new reagents behave in terms of analytical and diagnostic accuracy compared with the previous version of the assay.

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ADVICE FOR MEMBER COMMENTS

Appendix Number and Title: 1.1.4.4. Measurement uncertainty

Country making the comments

Date

NB: The Biological Standards Commission is particularly interested in OIE Member Country opinion on this appendix (and its accompanying chapter and six other appendices): are the documents clear and useful? If not, how they could be improved?

It would be appreciated if the following guidance notes are followed in making a reply:

1. *Comments may be general or specific, but specific comments are usually more valuable. General comments should be such that some conclusion and action can be taken in response to them. For example, it is of little help to make statements such as "This test is no longer used in our laboratory", without indicating the reasons and what test is used instead.*

2. *Specific comments should be identified, by indicating the line number in the text, to facilitate the making of editorial changes.*

3. *The pointing out of typing or technical errors is welcome, but the correct word or figure should be indicated. For example, it is of little help to say that 0.8 M is too high, if the preferred value is not indicated.*

4. *Bear in mind that the introductory chapters are not intended to be exhaustive, and indeed none of the chapters can give a completely comprehensive cover of the subject, otherwise the* Terrestrial Manual *would be too long. However, assistance in indicating priorities is always helpful.*

5. *The* Terrestrial Manual *is intended for world-wide use. The chapters need to reflect the development of new technology, but the old established methods, usually requiring less sophisticated apparatus, have to be retained. New technology should not be described in detail until it has gained wide acceptance as a reliable method.*

General Comments

Specific Comments (*add continuation sheets if required*)
line:

APPENDIX 1.1.4.4.

MEASUREMENT UNCERTAINTY

INTRODUCTION

As the measurement process for detection of an analyte in a diagnostic sample is not entirely reproducible, there is no exact value that can be associated with the measured analyte. So the result is most accurately expressed as an estimate together with an associated level of imprecision. This imprecision is the measurement uncertainty (*MU*). *MU is limited to the measurement process. 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 Chapter 1.1.4/5., Section B.2.a*).

1. The necessity of determining MU

Estimation of MU is a requirement based of international quality standards such as ISO/IEC 17025-2005, *General requirements for the competence of testing and calibration laboratories* (ISO/IEC 17025). National accreditation bodies assess MU estimates for 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. Suitable statistical measures to express MU are mean values ± 2 standard deviations (95% CI), relative standard deviation (RSD = SD / mean of replicates), which is expressed as the absolute value of the coefficient of variation, and the coefficient of variation (CV = standard deviation of replicates / mean × 100), which is expressed as a percentage. RSD is expressed as a fraction, but more usually as a percentage (%RSD) which is synonymous with coefficient of variation. The concept of MU does not apply to strictly binary results (positive or negative).

a) 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 replicates from a minimum of three (preferably five) samples representing analyte activity within the operating range of the assay (see Chapter 1.1.4/5., Sections A.2.f and B.2.a, and Appendix 1.1.4.7., Section 3.a). The variation in replicate results can be expressed as standard deviations, confidence intervals, or other possible options. The point here is that repeatability studies 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 Chapter 1.1.4/5., Sections A.2.g and B.6.a, and Appendix 1.1.4.7., Section 1.d). 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 of a test procedure. The point here is that quality controls are a monitor of assay precision and provide evidence that the assay is or is not performing as expected. In order 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.

The variation of the 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 amount of variation of 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 being made with respect to whether the test result will be considered as either positive, negative, or inconclusive (as will be described in the following example). For determining MU, low positive samples, like those used in repeatability studies or as the low positive control, are most appropriate. The rationale being that MU, which is a function of assay precision, is most critical at decision-making points (i.e. thresholds) which are usually near the lower limit of detection for

the assay. In this Appendix 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.

b) Example of MU calculations

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 Chapter 1.1.4/5., Section B.3.d).

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 positive control sample was used to calculate MU at the cut-off level.

i) 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 positive control serum, the relative standard deviation (RSD, or coefficient of variation (CV), if expressed as a percentage), provides a convenient transformation:

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

To simplify assessment, the transformed result is regarded as the assay output result (X). In the case of this example, a competitive ELISA, results are "normalised" (as defined in Chapter 1.1.4/5., Section A.2.h) 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 now referred to as the percentage inhibition or PI value. So for the low positive control serum (OD_L), the transformation is:

$$PI_L = 100 \times [1- \{OD_L / OD_N\}]$$

The relative standard deviation becomes:

$$RSD\ (PI_L) = SD(PI_L)/PI_L$$

ii) Example

A limited data set for the AI competitive ELISA example is shown below. Ideally in the application of this "top down" method, a large 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).

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

Mean = 56.3; Std Dev (SD) = 7.9; Assays (*n*) = 10; RSD = SD/Mean: 0.14

iii) Calculating uncertainty

From the limited data set,

RSD (PIL) = 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 particular level of confidence. Expanding the uncertainty is done by multiplying the RSD (PIL) by a factor of 2; this allows the calculation of approximate 95% confidence levels around the threshold value, assuming normally distributed data.

U(95%CI) = 2 × RSD = 0.28

This estimate can then be applied at the threshold level (in this case at PI = 50%)

95% CI = 50 ± (50 × 0.28) = 50 ± 14%

iv) Interpretation

Any positive result (PI > 50%) that is less than 64% is not positive with 95% confidence. Similarly, a negative result (PI < 50%) that is not less than 36% is not negative at the 95% confidence level. This zone of lower confidence for interpretation may correlate with the "grey zone" or "inconclusive/suspect zone" for interpretation that should be established for all tests (Greiner *et al., 1995*).

2. Other applications

The top-down approach should be broadly applicable for a range of diagnostic tests including molecular tests. For the calculation of test using a typical two-fold dilution series for the positive control (such as in a VNT, CFT, HI etc.), geometric mean titre (i.e. mean and 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) titre, and finally transformed 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 Subcommittee for Animal Health Laboratory Standards (SCAHLS) has produced worked examples for a number of diagnostic tests, which are available at the SCAHLS webpage at:

http://www.scahls.org.au/policyguidelines/Worked_MU_examples.doc

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 documents are available from the National Pathology Accreditation Advisory Group and Life Science. The central document to MU is the Guide to the expression of uncertainty in measurement (GUM), ISO/IEC Guide (1995).

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ADVICE FOR MEMBER COMMENTS

Appendix Number and Title: 1.1.4.5. Statistical approaches to validation

Country making the comments

Date

NB: The Biological Standards Commission is particularly interested in OIE Member Country opinion on this appendix (and its accompanying chapter and six other appendices): are the documents clear and useful? If not, how they could be improved?

It would be appreciated if the following guidance notes are followed in making a reply:

1. *Comments may be general or specific, but specific comments are usually more valuable. General comments should be such that some conclusion and action can be taken in response to them. For example, it is of little help to make statements such as "This test is no longer used in our laboratory", without indicating the reasons and what test is used instead.*

2. *Specific comments should be identified, by indicating the line number in the text, to facilitate the making of editorial changes.*

3. *The pointing out of typing or technical errors is welcome, but the correct word or figure should be indicated. For example, it is of little help to say that 0.8 M is too high, if the preferred value is not indicated.*

4. *Bear in mind that the introductory chapters are not intended to be exhaustive, and indeed none of the chapters can give a completely comprehensive cover of the subject, otherwise the* Terrestrial Manual *would be too long. However, assistance in indicating priorities is always helpful.*

5. *The* Terrestrial Manual *is intended for world-wide use. The chapters need to reflect the development of new technology, but the old established methods, usually requiring less sophisticated apparatus, have to be retained. New technology should not be described in detail until it has gained wide acceptance as a reliable method.*

General Comments

Specific Comments (*add continuation sheets if required*)
line:

APPENDIX 1.1.4.5.

STATISTICAL APPROACHES TO VALIDATION

INTRODUCTION

The choice of statistical methods for analysis of test validation data from laboratory experiments and from evaluation of field-based samples depends on considerations for experimental design and sample selection (*source, number of samples, number of replicates of tests, etc.*)*. Specific guidance about the "best approach" should be made in consultation with a statistician and should be done during the design phase before validation studies commence.*

For brevity, this annex considers commonly-used approaches for validation of a candidate test and hence, does not consider all statistical methods that might be used in practice. Methods are described to estimate the precision of an assay when repeated multiple times (*repeatability and reproducibility*), *analytical characteristics* (*analytical sensitivity and specificity*) *and diagnostic characteristics* (*e.g. diagnostic sensitivity* [*DSe*] *and specificity* [*DSp*]*, and area under the receiver-operating characteristic curve of an assay*) *used to detect an analyte in individual animals. Similar principles apply when tests are used to detect the same analyte in naturally or artificially-created sample pools from animals in aggregates* (*e.g. herds or flocks*). *In this case, the study unit is the aggregate rather than individual animals.*

Definitions of scales of measurement:

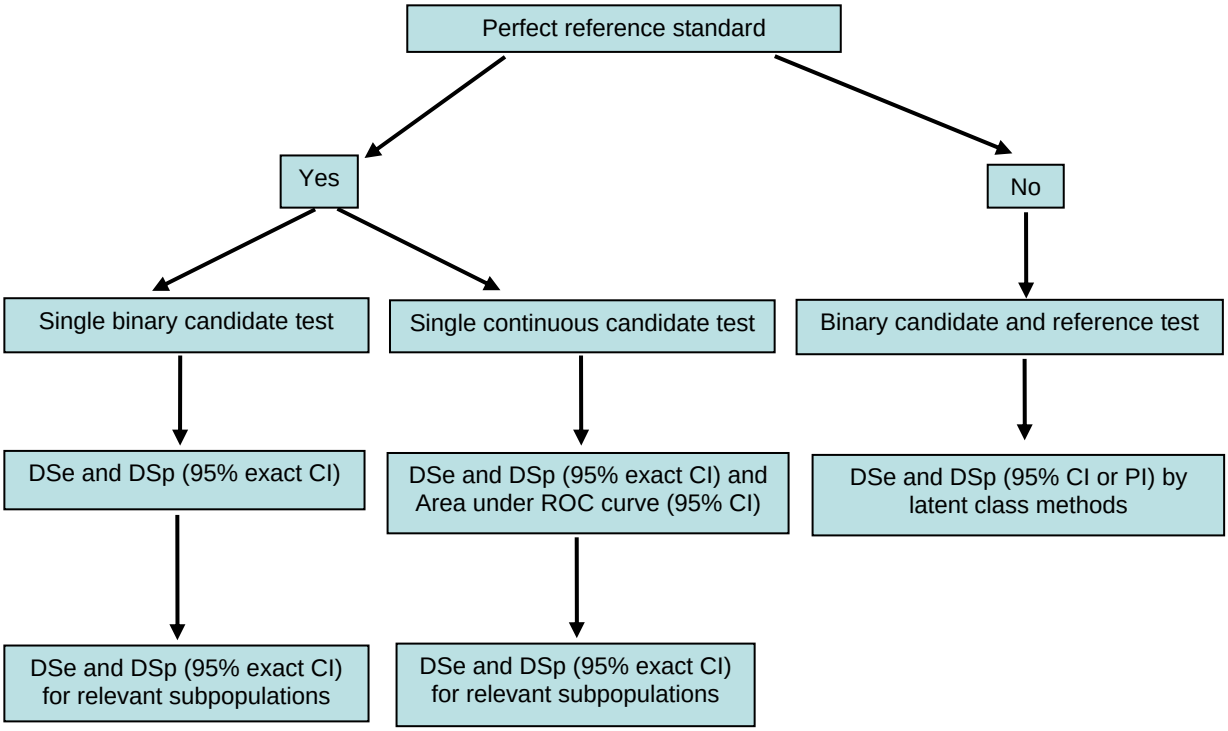
Binary (dichotomous): Either positive or negative because that is how the test result is presented, or positive/negative at a selected threshold value when results are measured on an ordinal or continuous scale.

Ordinal: Measured on a scale with discrete values where higher values typically indicate more analyte, e.g. serum virus neutralisation titres.

Continuous: An infinite number of measured values are theoretically possible, depending on the measurement system, e.g. ELISA optical density values, cycle threshold values in real-time PCR.

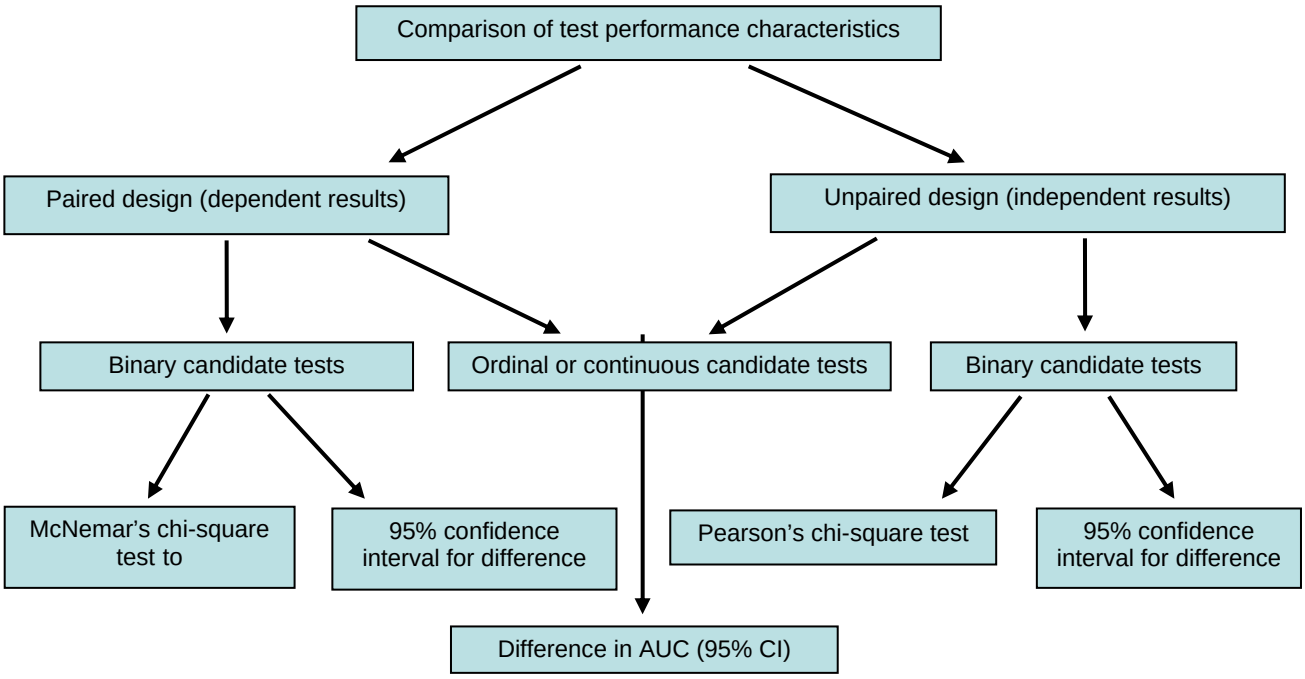
Statistical methods differ depending on whether a single or multiple tests evaluated, their scales of measurement (*binary, ordinal, or continuous*)*, whether independent or dependent* (*paired*) *samples are used, and whether there is a perfectly accurate reference standard test method* (*often termed a gold standard test*) *for comparison* (*Wilks, 2001*). *Flow charts to guide selection of statistical methods are in Figures 1 and 2.*

The adequacy of the design of the study and statistical analysis may not always be reflected in the quality of reporting, so test developers and evaluators are encouraged to follow the STARD (***Sta****ndards for* ***R****eporting of* ***D****iagnostic Accuracy*) *checklist to ensure complete reporting of all relevant information in validation studies of infectious diseases in animals. Uninterpretable, indeterminate and intermediate test results, if present, should be accounted for in reports of all analyses* (*Bossuyt, 2003*)*.*



Abbreviations: DSe = diagnostic sensitivity; DSp = diagnostic specificity; ROC = receiver operating characteristic; CI = confidence interval; PI = probability interval.

Figure 1. *Flow chart for suggested methods of statistical analysis when a single candidate test is evaluated with and without a perfect reference standard.*



Abbreviation: AUC = area under the receiver operating characteristic curve

Figure 2*. Flow chart for suggested methods of statistical analysis when sensitivities and specificities, and areas under the receiver-operating characteristic curve of multiple tests, are evaluated with a perfect reference standard. Ordinal and continuous data should be analysed in their original form and as binary results at the recommended thresholds. Analyses should be done for both*

sensitivity and specificity where these data are available.

1. Assay precision within a single laboratory

An assessment of within-laboratory precision of an assay (often termed *repeatability*) requires that a minimum of 3 (and preferably 5) samples having analtye concentrations within the operating range of the assay are tested in replicate by a single operator using a single test-kit lot or batch. Typically these runs will be on the same day but separate days are also possible. Use of 3 or 4 replicates, rather than 2, is encouraged since it better captures the inherent variability in within-run assay results. Because of cost considerations, use of more than 2 replicates may not be feasible for all assay types (e.g. nucleic-acid detection). As described in the chapter, between-run variation can be evaluated in multiple runs (approximately 20) involving 2 or more operators on at least 5 separate days.

Statistical uncertainty about diagnostic performance parameters, e.g. DSe and DSp, should be presented as confidence intervals (CI). Typically, a 95% CI is used and its width (precision of the estimated value) depends strongly on the sample size used for parameter estimation

a) Continuous outcomes

For continuous outcomes, the simplest approach is to estimate the standard deviation (SD) of replicates of a set of samples representing the operating range of the assay. These results initially should be evaluated in a scatter plot or diagram of the mean of the replicates plotted against the SD. For assays, where the SD is proportional to the mean, the within-sample coefficient of variation (CV) is often calculated. CV is often used even when proportionality doesn't exist. In this case, the CV should be reported for levels of the target analyte (e.g. low, moderate and high). This is necessary because it is a common finding that CV is often larger when the concentration of target analyte is low. In general, an estimate of uncertainty in the CV values (e.g. 95% confidence interval) should also be calculated. Where the CV values are fairly constant over the range of test values, this can be done using the results of all samples. Where CV differs according to analyte concentration, separate 95% confidence intervals should be calculated for each analyte category based on the number of samples tested at each level.

$$CV = \frac{\text{SD of replicates}}{\text{Mean of replicates}}$$

Where: CV = Coefficient of variation
SD = Standard deviation

b) Binary outcomes

In general, quantitative results should be used for evaluation of assay precision when data are available in that form, even though results might be dichtomotised for reporting purposes. For inherently binary tests which yield positive or negative results, the kappa statistic can be used to quantify the agreement of test results beyond chance (Fleiss, 1981). Kappa ranges from 0 (no agreement beyond chance) to 1 (perfect agreement beyond chance) but there is much conjecture about how kappa values should be interpreted (Fleiss, 1981; Landis & Koch 1977). Better agreement is typically expected when test results are well away from the cut-off points and hence, some samples with intermediate/suspicious values should be tested to avoid overly optimistic assessments of agreement. A weighted version of kappa for ordinal results (e.g. negative, suspicious, and positive) can be used to recognise that a large discrepancy (e.g. two category difference) is more serious than a smaller discrepancy (e.g. one category difference). Ninety-five per cent CI should be reported for unweighted or weighted estimates of kappa (Fleiss, 1981)

Table 1. *Examples of Kappa calculations for binary outcomes*

Example 1: Kappa calculation based on duplicate results classified as positive or negative

Test result	***Positive***	***Negative***
Positive	90	5
Negative	10	95
	100	100

Kappa = 0.85 (95% CI = 0.78 to 0.92)

Example 2: Kappa calculation based on duplicate results classified into three categories (positive, negative or suspicious)

Test result	***Positive***	***Suspicious***	***Negative***
Positive	80	10	10
Suspicious	15	75	10
Negative	5	15	80
	100	100	100

Kappa = 0.68 (95% CI = 0.61 to 0.75). Weighted kappa = 0.70. (95% CI = 0.61 to 0.79)

c) Robustness

Robustness of an assay is a measure of its capacity to remain unaffected by small but deliberate variations in the test method parameters and provides an indication of its reliability during normal usage. Lack of robustness is evident when the width of confidence intervals for precision estimates within and/or between runs is wider than expected. For candidate assays that have undergone extensive optimisation studies, the best possible combination of parameters may still yield an assay that is inherently less precise than other assays of the same type (such as ELISA). Experience suggests this reflects a method bias (reagent deficiency such as reduced affinity of a monoclonal antibody-based conjugate). The method bias may be different from day to day and, when viewed from the perspective of repeated runs of the assay, it has a repeatable component associated with it which may contribute to a reduced precision estimate for between-run variability. A more in-depth discussion is described, and methods for measuring robustness are cited, in the Validation Chapter 1.1.4/5.

2. Assay precision among laboratories

Assay precision will vary according to routine implementation e.g., different operators, different test sites, using different kit lots, or on different days. Most commonly, the term *reproducibility* is applied to assessment of precision of the selected assay in multiple laboratories. Factors held constant should be described to allow interpretation of results in context to the actual testing situation. Reproducibility studies can be done independently of or in association with repeatability studies but should be done in a blinded fashion.

Statistical methods for analysis of studies of assay precision among laboratories are similar to those used for assessment of within-laboratory precision. However, as part of an among laboratory study, it might be considered important to assess and rank variability in test results from multiple sources (often termed a class). For example, if a study was designed to test an assay in 3 laboratories each using 2 highly-trained technicians and running the samples in duplicate on 2 kit lots, each test sample would be tested 24 times. The selected factors (laboratory, technician, kit lot, replicate result) can be considered to be fixed or random depending on how they are selected and whether they are representative of the target population. For this study design, variance components can be estimated for each class (example is Dargatz *et al.,* 2004) and the intraclass (intracluster) correlation coefficient (ICC) can be estimated as a measure of the similarity of sample results (Bartlett and Frost, 2008).

a) Ruggedness

Ruggedness is considered synonymous with reproducibility, and is expressed as random measurement error between-laboratories, as outlined in the preceding section. The participating laboratories would conduct the same test method in similar laboratory environments, using the same panel of samples, reagents, and instrumentation (when possible). The data would be evaluated for ruggedness as described in the preceding paragraph.

Intraclass correlation coefficient represents the similarity or correlation of any 2 measurements made on the same sample. The ICC takes values between 0 and 1 with values closes to 1 indicating minimal measurement error. Conversely, values close to 0 indicate a large amount of measurement error.

b) Beyond a ruggedness evaluation: when a technical modification of the test method is required

An exceptional situation may arise when an assay that has been validated for use in a controlled laboratory environment is being considered for use in a very different environment (such as a pen-side application). Because of the more extreme changes, for example severe temperature fluctuations which often occur at pen-side, it would be expected that the two tests will behave very differently in their different environments. In fact, rather than random measurement error which applies to the assessment of within or among laboratory measurement error, it is anticipated that the values in such a study likely would be interpreted as a systematic measurement error, which would be the case if the values provide an over- or under-estimate

of the true value. For the example of a test run on split samples pen-side and in a laboratory, the mean of the differences between the pen-side value and the within laboratory value (true value) for the same sample should be reported with a 95% confidence interval. If the 95% confidence interval excludes zero, there is evidence of systematic deviation of test results when used pen-side.

When such a systematic deviation in test results occurs, the pen-side test results do not comport with the laboratory-based validated assay in terms of being comparable. To validate the pen-side assay, either it is subjected to a "technical modification" that is then evaluated in a methods comparison study (Appendix 1.1.4.6. Comparability of assays after minor changes in a validated test method) or a full re-validation of the pen-side application is required.

3. Analytical sensitivity (ASe, synonym = limit of detection: LOD)

Analytical sensitivity can be estimated using a dilution to extinction (DTE) approach in which serial dilutions of a known quantified amount of target analyte are made into the appropriate sample matrix (Philip *et al.,* 2006). This known quantified amount might be from an in-house or national/international reference standard or a field sample whose analyte concentration has been determined. Parallel runs of a comparison standard can be done but are not essential, unless the study is one in which a minor change in a validated assay is being compared with the original validated assay.

The DTE approach can be used if the analyte is measured qualitatively or quantitatively in which case the test result is reclassified as positive or negative. A minimum of four dilutions are required with a least one dilution yielding a 100% detection and one dilution yielding 0% detection. The LOD may be expressed as the endpoint titer, which is the next-to-the-last dilution in a dilution series where the last dilution is the one in which the analyte can no longer be detected. A more exacting inference of LOD is expressed as the last dilution at which a certain percentage of the replicates of a dilution are positive for the analyte. For instance, if a 50% LOD is chosen, it would be the dilution wherein at least one-half of its replicates remain positive for the analyte. Alternatively, if a 95% LOD is chosen, it would be the dilution wherein 19 of 20 replicates for that dilution would remain positive for the analyte. The important point is not whether you choose 50%, 95%, or n%, but that the chosen percentage for the LOD is specified. The greater the number of replicates for each dilution, the stronger the estimate of LOD. Recommendations of the working group on microbiological methods (Philip *et al.,* 2006), suggest that a 50% endpoint be estimated. This is analogous to the 50th percentile of the lethal dose (LD_{50}) of viral cytopathic effects in cell culture. The LD_{50} can be estimated using the Spearman-Karber non-parametric approach, or by logistic regression or probit analysis (Philip *et al.,* 2006). In all cases, point estimates and 95% CI should be reported. If a more conservative estimate is needed, then the 95% endpoint can be calculated. This is interpreted as the concentration of the analyte in the sample matrix that would be detected with high statistical certainty (95% of the time).

4. Analytical specificity (ASp)

Analytical specificity can be described in at least three distinctive ways: selectivity, exclusivity (synonym: cross-reaction profile), and inclusivity (as described in Chapter 1.1.4/5.). The latter two measures should be reported on a per lineage, isolate, species or genus basis, as appropriate for the target analyte and the intended purpose of the test. For screening tests, a broader and more inclusive specificity is required than for a confirmatory test which may distinguish between isolates that, for instance, vary in pathogenicity. Because the choice of related organisms is subjective and often dependent on the types and numbers of samples, the exclusivity result should be reported qualitatively, e.g. the percentage of related agents that cross-reacted in the assay with a listing of potential cross-reacting agents that were evaluated. Similarly, inclusivity is reported as a percentage of the serovars, strains, genera and species detected by the assay, as appropriate for the target analyte.

5. Diagnostic sensitivity (DSe) and diagnostic specificity (DSp)

These parameters can be estimated when the reference or comparison method is perfectly sensitive and specificity or when the reference standard is imperfect. In general, most ante-mortem reference standards in common use in diagnostic laboratories are imperfect and hence, necropsy with testing of multiple tissues by ancillary tests such as culture and/or histopathology often is necessary if the results of the reference standard are to be considered to be the truth. For most test validation studies in animal health, this option is not feasible except for a limited number of samples.

a) DSe and DSp with a perfect reference standard

The candidate test may yield results on binary (dichotomous), ordinal (e.g. titre) or continuous scales. For the latter two scales, results need to be dichotomised before DSe and DSp can be calculated, i.e., a cut-off (threshold) needs to be established. Exact binomial 95% confidence limits are recommended for DSe and DSp (Greiner & Gardner, 2000) because the normal approximation may not yield valid confidence intervals when parameter estimates are close to 1, When the reference standard is not applied to all candidate test

results (partial verification), corrected estimates of DSe and DSp should be made as described in Greiner & Gardner (2000).

For assays yielding ordinal (e.g. titre values) or continuous results (e.g. matrix-to positive ratios in an ELISA), estimates of DSe and DSp should be complemented with estimates of the area under the receiver-operating characteristic (ROC) curve. ROC analysis provides a cut-off-independent approach for evaluation of the global accuracy of a test whose results are measured as ordinal or continuous values. The area under the ROC curve provides a single numerical estimate of overall accuracy ranging from 0.5 (useless test) to 1 (perfect test). The main justification for ROC analysis is that cut-off values for test interpretation may change depending on the purpose of testing (e.g. screening versus confirmation) and with the prevalence of infection, the costs of test errors, and the availability of other tests. Detailed descriptions of ROC analysis are presented elsewhere (Gardner & Greiner, 2006; Greiner *et al.,* 2000; Zweig & Campbell, 1993). When multiple ordinal or continuous tests are compared, the difference in the area under the curve with a 95% CI should be calculated. Methods for calculating differences vary for independent and dependent samples and are implemented in many statistical programs (Gardner & Greiner, 2006). Examples of a dot diagram for results of a single ELISA and a ROC curves for 2 ELISAs are shown in the Figures 3 and 4.

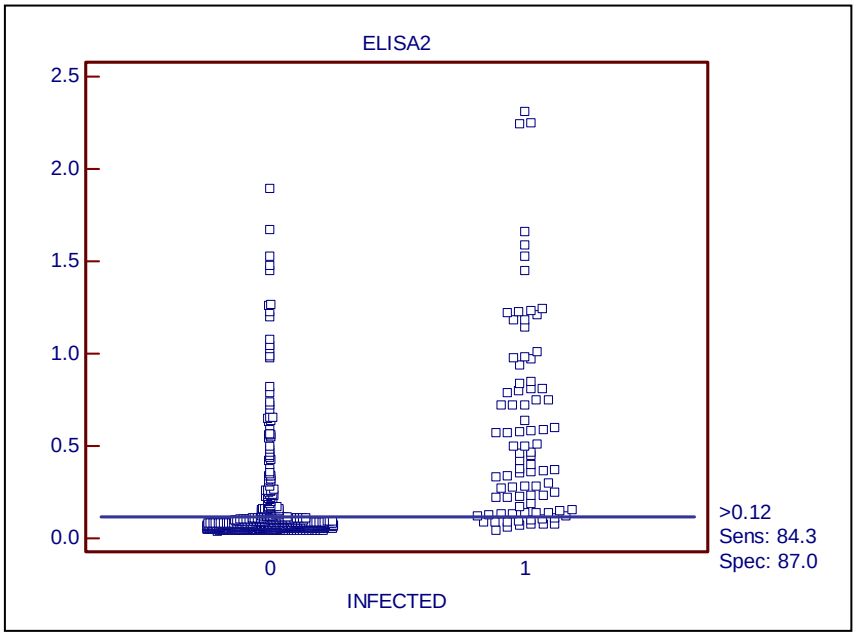


Figure 3. *Dot diagram of ELISA results for non- infected (code = 0) and infected (Code = 1) animals.*

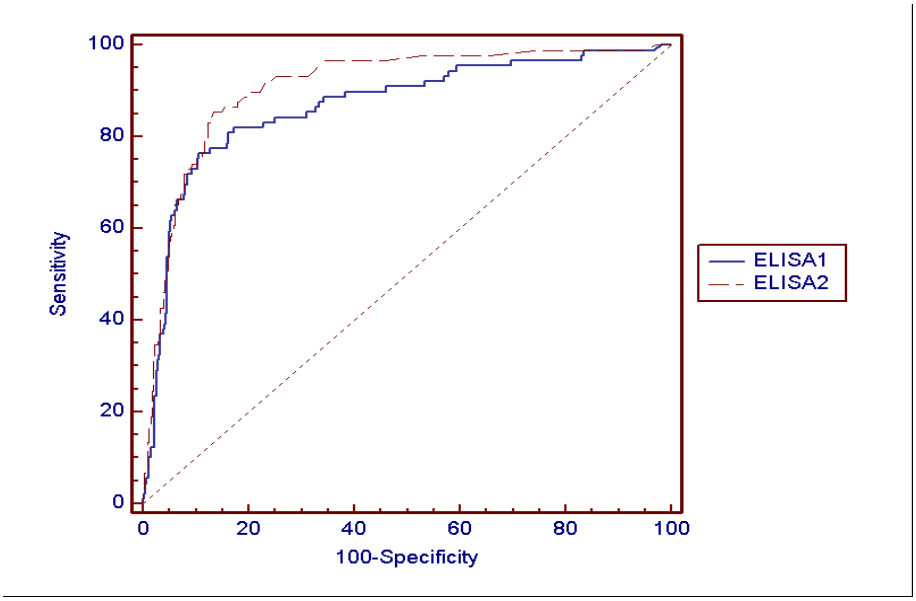


Figure 4. *Receiver-operator characteristic curve for two ELISAs.*

b) Comparison of DSe and DSp estimates for 2 tests with a perfect reference standard

Often, investigators might wish to compare DSe values in subpopulations of infected animals, e.g. clinically versus subclinically infected, or DSp values in different geographical areas. Since these are independent samples, the comparisons can be made statistically be Pearson's chi-square test for homogeneity. Alternately, separate 95% CI and a 95% CI for a difference in two proportions can be calculated. When the DSe (or DSp) of two tests are compared on the same set of infected (or non-infected) samples in a paired design, the test results are no longer independent. Statistical methods such as McNemar's chi-square can be used to test the hypothesis of equal sensitivities (specificities) when testing is done on the same samples.

c) DSe and DSp without a perfect reference standard

Recent advances in statistical methodology, namely the development of latent class ("no-gold-standard") methods, now allow investigators to liberate themselves from the restrictive assumption of a perfect reference test and estimate the accuracy of the candidate test and the reference standard with the same data (Enoe *et al.,* 2000; Hui & Walter, 1980). The method can be applied when three conditionally independent tests are run on the same samples from a single population; however, the constraint of independence of three tests may be difficult to achieve in practice. Hence, the most commonly-used approach is to run two tests on all samples from two populations because it is less costly and assumptions of conditional independence may be more reasonable.

Maximum likelihood (ML) and Bayesian methods can be used for estimation of DSe and DSp in the absence of a perfect reference test. Bayesian approaches are especially suited to situations where prior information is available about test performance and when the estimation problem is not identifiable i.e., when there is no unique set of ML parameter values (Branscum *et al.,* 2005). The WinBUGS software allows easy implementation of Markov-chain Monte Carlo methods for Bayesian estimation (Lunn *et al.,* 2000) and simple ML analyses can be done using a web-based interface (Poulliot *et al.,* 2002). Prior information about model parameters used in the Bayesian analyses may affect the final estimates depending on the relative strength of evidence provided by the priors (level of prior uncertainty) and the data (uncertainty attributable to finite sample sizes). Therefore, the sources of prior information must be well documented in Bayesian analyses.

Maximum likelihood – a statistical algorithm which estimates the most likely values for the parameters of interest based on the value of likelihood function for the data.
Bayesian methods differ from maximum likelihood because they incorporate relevant prior information or knowledge about one or more tests in addition to the likelihood function for the data. With large sample size, maximum likelihood and Bayesian methods will yield similar inferences.

Standards for use of latent class methods have been recently proposed for infectious diseases of animals (Mintiens *et al.,* 2010) but these methods cannot correct for biases inherent in poorly designed studies. The methods should be used carefully and include a thorough evaluation of underlying assumptions (e.g. conditional dependence, constant sensitivity and specificity across populations, and differing prevalence) including the effects of use of the selected prior distributions on posterior inferences, and convergence of Markov chains in a Bayesian analysis (Toft *et al.,* 2005).

d) Comparison of DSe and DSp estimates for two tests without a perfect reference standard

If two tests are evaluated on all sampled units in two populations in a maximum likelihood analysis, estimates of DSe and DSp and associated 95% CI can be obtained for both tests. If a Bayesian approach is used in WinBUGS to analyse the data, the difference in sensitivities (specificities) can be readily estimated and the probability that the sensitivity (specificity) of one test exceeds the other can be estimated with a STEP function. Examples of application of this approach to toxoplasmosis data in pigs are presented in Gardner *et al.* (2010).

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ADVICE FOR MEMBER COMMENTS

Appendix Number and Title: 1.1.4.6. Comparability of assays after minor changes in a validated test method

Country making the comments

Date

NB: The Biological Standards Commission is particularly interested in OIE Member Country opinion on this appendix (and its accompanying chapter and six other appendices): are the documents clear and useful? If not, how they could be improved?

It would be appreciated if the following guidance notes are followed in making a reply:

1. *Comments may be general or specific, but specific comments are usually more valuable. General comments should be such that some conclusion and action can be taken in response to them. For example, it is of little help to make statements such as "This test is no longer used in our laboratory", without indicating the reasons and what test is used instead.*

2. *Specific comments should be identified, by indicating the line number in the text, to facilitate the making of editorial changes.*

3. *The pointing out of typing or technical errors is welcome, but the correct word or figure should be indicated. For example, it is of little help to say that 0.8 M is too high, if the preferred value is not indicated.*

4. *Bear in mind that the introductory chapters are not intended to be exhaustive, and indeed none of the chapters can give a completely comprehensive cover of the subject, otherwise the* Terrestrial Manual *would be too long. However, assistance in indicating priorities is always helpful.*

5. *The* Terrestrial Manual *is intended for world-wide use. The chapters need to reflect the development of new technology, but the old established methods, usually requiring less sophisticated apparatus, have to be retained. New technology should not be described in detail until it has gained wide acceptance as a reliable method.*

General Comments

Specific Comments (*add continuation sheets if required*)
line:

APPENDIX 1.1.4.6.

COMPARABILITY OF ASSAYS AFTER MINOR CHANGES IN A VALIDATED TEST METHOD

INTRODUCTION

Technology advancements, available resources, and time constraints often dictate the need for minor changes in validated testing procedures. Commonly encountered examples include substitution of one marker dye or reagent for another in enzyme-linked immunosorbent assay (*ELISA*), *polymerase chain reaction* (*PCR*), *and immunohistochemistry procedures, or transition from manual systems to robotics for sample processing and handling. In contrast, a change in target gene in a PCR assay or a change from indirect to a competitive ELISA format would be considered a major change warranting complete or partial revalidation of the assay.*

When minor changes are introduced into an assay that investigators believe will not biologically affect assay performance, a rigorous and well designed "methods comparison study" is needed to determine whether the assay, when used with the minor change (*hereafter termed the substitute method*)*, is comparable to, or as fit for the intended use, as the validated process. The term "equivalence" or "equivalency" has historically been used in some diagnostic laboratories for such studies. However, to avoid confusion with the statistical meaning of the term and the associated sample size implications, the term is not used again, nor implied, in this appendix.*

Although the ideas presented in this appendix are applicable to many assay procedures and to both foreign and endemic diseases, the examples deal specifically with PCR for viral diseases. This appendix provides details of experiments that are useful for determining if the methods are comparable for minor changes in a validated PCR process. Ideally, the methods comparison study should include diagnostic samples in addition to laboratory-spiked samples that were not conducted in the target sample matrix. Guidance about specific sample numbers and experimental design should be made in consultation with a statistician prior to starting the studies.

In this appendix, experiments and examples are described for five possible scenarios consistent with Stage 1 validation (*see Chapter 1.1.4/5.*)*:*

1. *Comparison of methods for limit of detection* (*analytical sensitivity*)
2. *Comparison of methods for inter- and intra-assay precision* (*repeatability*)
3. *Assessment of operator effects on within-laboratory assay precision*
4. *Comparison of methods using diagnostic samples*
5. *Assessment of cross contamination*

Not all scenarios are needed in every methods comparison study. For example, an assessment of cross contamination would mostly only be necessary for changes in equipment. Similarly, methods assessment for diagnostic samples might be difficult or impossible for foreign animal diseases but much more feasible for endemic diseases.

For all experiments, a decision criterion is necessary to assess whether comparability has been achieved. Ideally, the criterion should be decided prior to experimentation and for transparency, the names and qualifications of the experts used to decide the criterion should be documented.

1. Comparison of methods for limit of detection (analytical sensitivity)

For most immunologic, enzymatic, or chemically-based assays, a substituted procedure, reagent, or technical approach will have a defined biologic role. Hence, an early step in a methods comparison study is to assess the altered assay reagent or process in the biologic context of the specific component of the assay. For example, if an altered extraction protocol is proposed to recover viral DNA prior to a real-time PCR analysis in a validated PCR

assay that had specified the DNA recovery procedure or reagents, the methods comparison study should focus on comparing the quantity of the DNA obtained. Typically, this will be evaluated through estimation of the lower limit of detection (LOD) of an assay, which in the case of PCR might be expressed as the amount of DNA or number of gene copies detected.

Various methods can be used to establish an LOD value. To obtain an accurate and precise estimate of LOD in the original validated assay, a two-stage experiment usually would have been conducted. This requires robust, expensive, and labor intensive experimentation with replications that allow appropriate statistical inference to be made about the LOD. However, less experimental rigor might be reasonable when determining if a minor change in the assay will result in an LOD that is anticipated to be comparable with that of the original validated assay.

This document interprets LOD to be the amount of analyte at which 95% of samples will be classified as positive. For a method comparison study to establish whether the two methods achieve comparable LODs, it is sufficient to choose an estimate of the LOD for the new method which is the dilution, in a dilution series of an analyte, in which all replicates in that dilution first become negative for the analyte.

The total amount of material required for the study will depend on the number of dilutions used in the experiment, the amount of material needed for each dilution for each method being compared, the amount of material needed to determine the titer, and the manner in which the dilution sequence is prepared. Prior knowledge from the validated method and isolate/serotype can be used to help determine the number of dilutions needed.

A decision criterion is needed to determine method comparability. For example, based on opinion of decision makers, it might be pre-specified that if the LOD of the substitute method, run side-by-side with the validated method, does not deviate more than a single tenfold dilution from the LOD observed in the validated assay, the methods will be deemed comparable with respect to the LOD. In Table 1, the LOD estimates for the two platforms differ by no more than 1 log unit and thus would be deemed comparable.

a) Design of the LOD experiment

A minimum of four and ideally six dilution sequences should be made but this decision should be informed by results of experiments from the initial validation study. The material should be aliquotted from each dilution into three replicates (“samples”) such that the three samples of each dilution will be run on each of two methods (Figure 1).

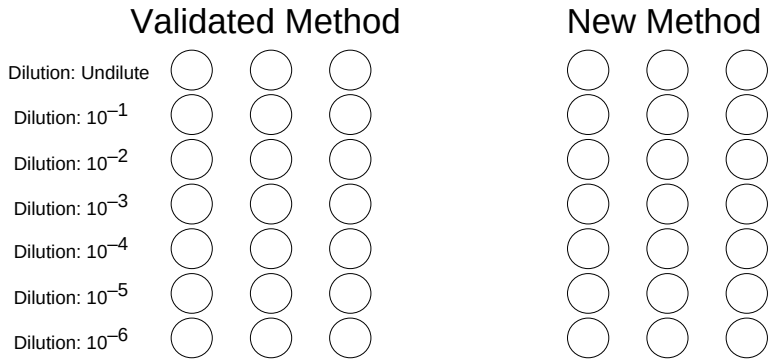


Figure 1. *Representative dilution series for an LOD experiment.*

b) Example of an experiment to determine LOD

An experiment was conducted to determine the limit of detection (LOD) for several isolate/chemistry/platform combinations for African swine fever (ASF) virus. Single and multiplex chemistries were tested using the Smart Cycler (SC), ABI7500, and ABI7900. Based on the observed data (Table 1 and graphical representations in Figure 2), it was determined that a lower cut-off value might be appropriate when using the multiplex chemistry. Table 1 provides the LOD for each isolate/chemistry/platform combination where a cut-off value of 37 was used for the multiplex chemistry and 40 for the singleplex chemistry. After the experiment was complete and the data analysed, there appeared to be a difference between the LOD estimates between the singleplex and multiplex chemistries when tested on the (SC). According to the decision criterion in the previous section, these chemistries would be deemed comparable based on their LODs.

Table 1. *LOD estimates (log_{10} - $TCID_{50}$ units) for different chemistries and platforms*

		Singleplex			***Multiplex***		
Isolate	$TCID_{50}$	SC	ABI7500	ABI7900	SC	ABI7500	ABI7900
1506	5.95	2.95	2.12	2.95	3.95	2.95	2.95
1512	6.36	3.36	3.36	3.36	4.36	3.36	3.36
1517	5.95	2.95	2.95	2.95	3.95	2.95	2.95
1520	6.58	3.58	3.58	3.58	4.58	3.58	3.58
1647	6.71	3.71	3.71	2.87	4.71	3.71	3.71
1663	6.52	3.62	3.52	3.52	4.52	3.68	3.62
2053	5.52	2.62	2.52	2.52	2.62	2.52	2.52
2379	5.21	2.21	2.21	2.21	3.21	2.21	2.21
3384	6.69	3.79	3.69	3.69	4.69	3.69	3.69

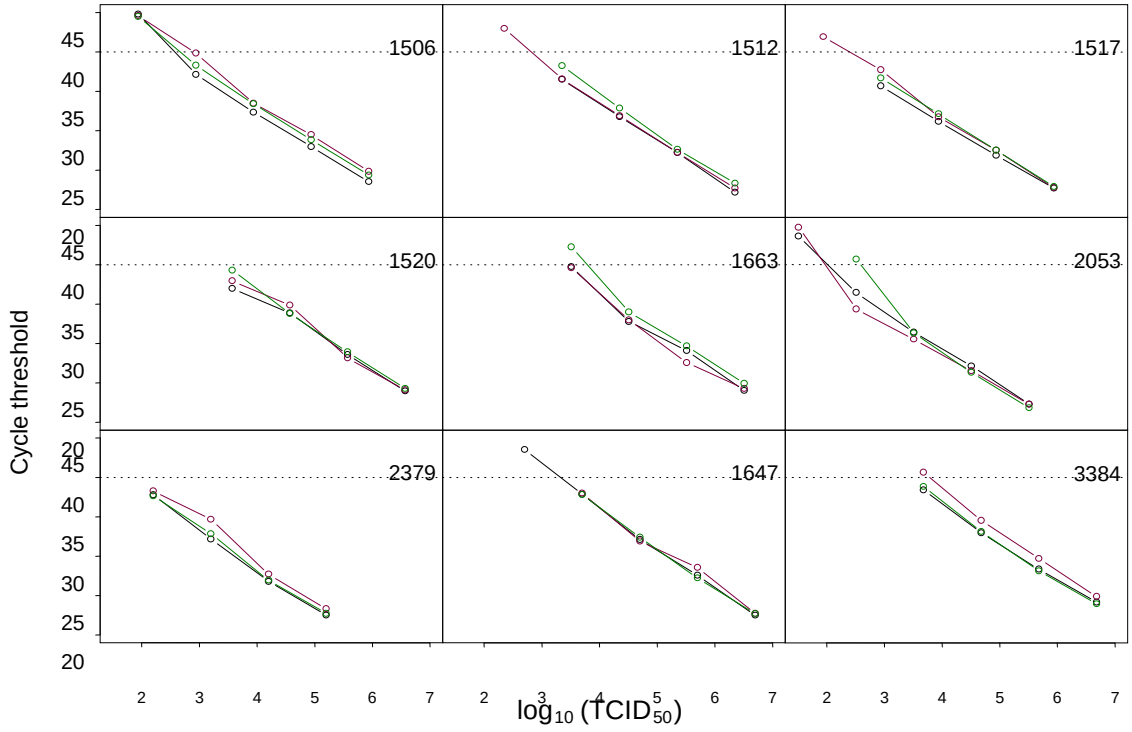


Figure 2. *Example of LOD data for nine isolates of African swine fever virus*

2. Comparison of methods for inter- and intra-assay precision (repeatability)

With any testing procedure, there are many sources of variability that can contribute to the precision of the final test result. This experiment is designed to estimate the sources of variability that are attributable to random differences between the same material on one plate (intra-assay precision) and variability that is associated with different runs of the assay procedure (inter-assay precision). The exact manner in which this experiment is conducted will depend on whether the minor change is related to the amplification process or the extraction process, as discussed separately below. In an attempt to isolate and estimate these sources of variability, the experiment must be conducted under strictly controlled conditions.

a) Comparison of two methods for variability attributable to the extraction process

Figures 3 and 4 represent the experimental procedure detailed in this section. A single operator should be assigned to conduct the entire experiment so that the variability between two different operators is not introduced into the experiment. Assessment of operator variability is described in Section 3, and should be assessed if there is a reason for concern that operator effects will be substantial.

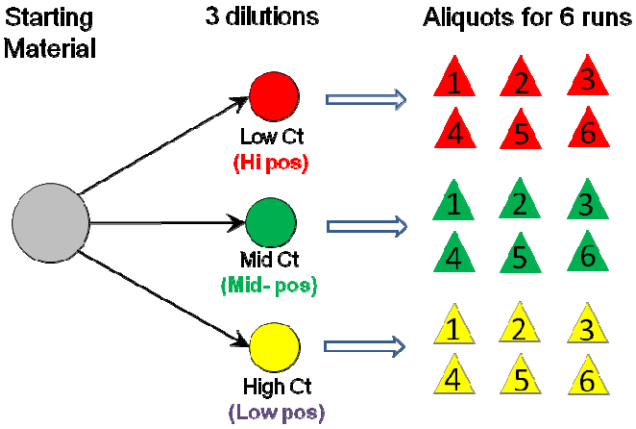


Figure 3. *Preparing the material for use in the precision study.*

Prior to beginning the experiment, the material should be prepared as shown in Figure 3. Initially, the amount of starting material needed should be determined. This will depend on the volume of material needed for a single sample for each of the methods, the manner in which the dilution series is created, and the amount of material required to determine the limit of detection by Ct values. The data obtained from the LOD experiment outlined above should help in determining the appropriate dilutions for this experiment.

From the starting material, three dilutions are made that span the range of cycle-threshold (Ct) activity in the assay (Figure 3). The low dilution (high concentration of analyte) should have a low Ct value and the medium dilution should have a Ct value near the middle of the Ct activity range in the assay system. The high dilution should have a high Ct value, which represents a concentration of analyte that is not detectable in any replicates of that dilution run in PCR (i.e. no inconclusive samples with values close to the cut-off).

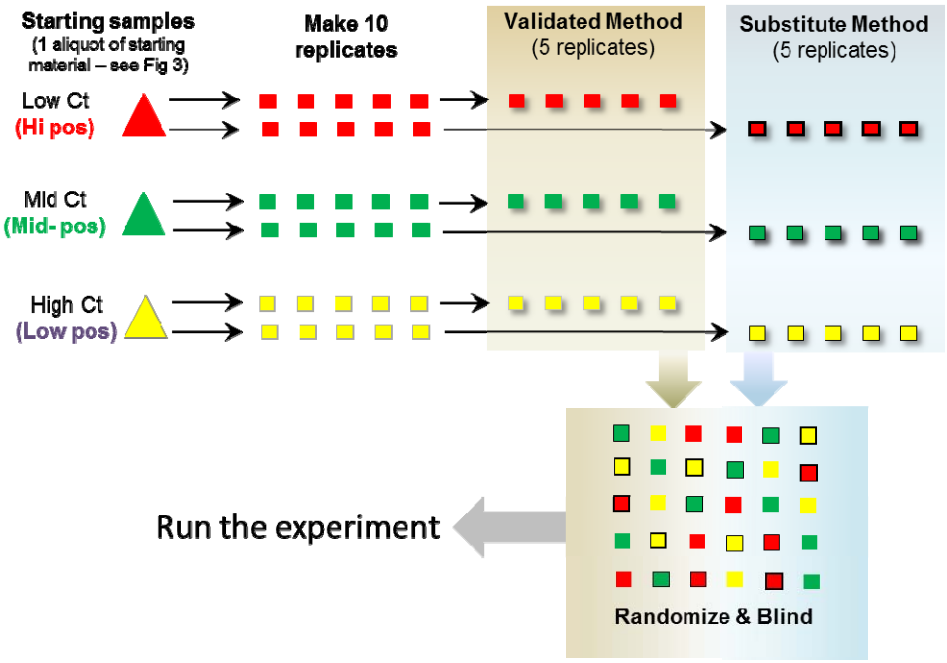


Figure 4. *Preparing the material that will be used in each run of the experiment.*

From each dilution, the material should be divided into six aliquots that will be stored for later use. Each aliquot should contain enough material to subdivide into five samples for testing on the validated method and five samples to be tested using the new method.

b) Experimental design to determine inter- and intra-assay precision ascribed to the extraction process

To estimate the inter- and intra-assay precision, six runs of the assay should be done where each run is conducted independently of all other runs. The independence can be achieved by conducting each run on a different day or conducting runs at different times during one day, such as one run in the morning and one in the afternoon. Each run will consist of performing the experiment using both methods in parallel (Figure 4).

One aliquot of each dilution is removed from storage and divided into ten replicates. Five replicates are run on the validated method and five replicates on the substitute method. If both methods require the same amount of material, the ten replicates will all be identical. Five replicates from each dilution (15 total samples) are assembled into the Validated Method Group. Similarly, the remaining 15 samples representing the remaining five replicates from each dilution will constitute the Substitute Method Group. The 15 replicates for each method should be randomised and labelled such that the operator is unaware of the sample content. The experiment is then repeated six times, using a new aliquot of each starting dilution (high-positive, mid-positive and low- positive as shown in Figure 3) in each run.

The extractions should be performed according to the appropriate procedure. Extracted samples can be stored until the amplification can be performed on all samples. All samples extracted from a single run (both methods) should be placed on the same PCR plate to minimise any source of variability that might affect the outcome other than those the experiment is designed to estimate (inter- and intra-assay precision).

Historically, intra- and inter-assay precision data were generated using a single dilution in every well on a plate (for a 96-well platform) and multiple plates were required. This proposed design is superior to the historically-used approach because more information can be obtained with a smaller number of samples.

i) Example 1: Manual versus robotic extraction procedures – FMD virus

Manual and robotic extraction procedures were compared for FMD virus to determine the inter- and intra-assay precision of the two procedures, run side-by-side. The data from the validated (manual) and new (robotic) procedures are given in Figure 5 and Table 2.

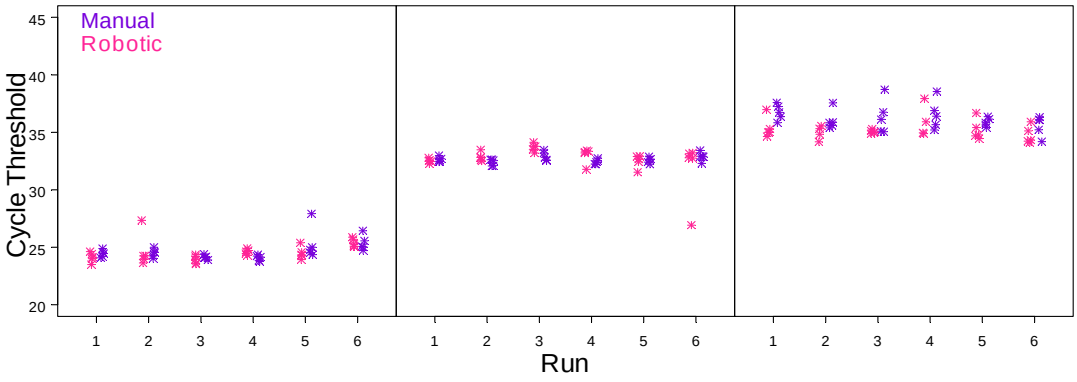


Figure 5. *Inter- and intra-assay precision data for FMD comparing robotic versus manual extractions.*

Table 2. *Example of inter- and intra-assay precision estimates (standard deviations) for FMD robotic versus manual extraction*

Method	Inter (SD)	Intra (SD)
Manual	0.28	0.73
Robotic	0.30	0.94

ii) Example 2. Inter- and intra-assay precision estimates: Avian Influenza high throughput extraction versus manual extraction.

A comparison of high-throughput (HT) extraction with the manual procedure was done to evaluate the efficiency of the HT procedure (Figure 6). Precision estimates expressed as standard deviations are provided in Table 3.

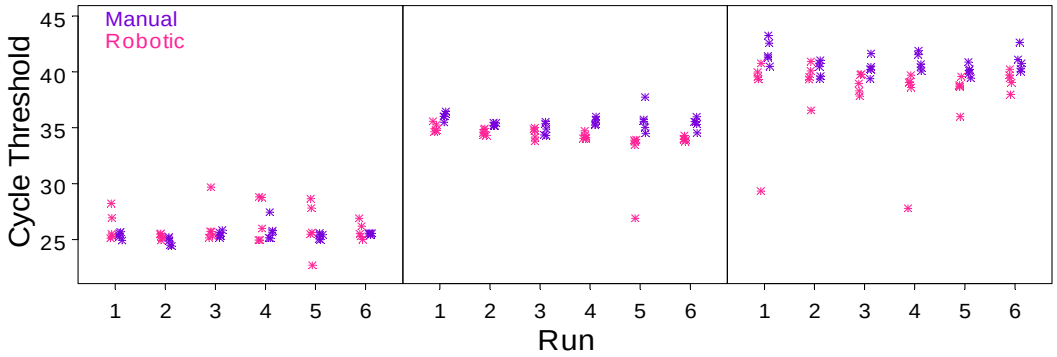


Figure 6. *Inter- and intra-assay precision data for AI high throughput extraction versus the manual extraction procedure.*

Table 3. *Inter- and intra-assay precision estimates – AI HT versus manual extraction*

Method	Inter (SD)	Intra (SD)
Manual	0.44	0.34
HT	0.50	0.50

c) Comparing methods for variability attributable to the amplification process

The steps for conducting this experiment are very similar to an experiment to compare extraction processes (Sections 2a and 2b, above) with the exception that the starting material is a pool of extracted RNA. The same kind of data analysis will be appropriate to assess differences in the amplification processes of the validated and substitute assays, as was done for comparison of extraction methods.

3. Assessment of operator effects on assay precision

For such an experiment, ten independent samples spanning the range of the assay should be used. Multiple individuals/technicians will perform the new process and usually it should be unnecessary to compare the results with those of the validated assay. It may be appropriate however, to perform this experiment to assess operator error when evaluation extraction methods, platform changes, and robotics.

4. Comparison of methods using diagnostic samples

The goal of this experiment is to determine if diagnostic samples behave in a similar fashion in the two testing methods (substituted and validated) without requiring acquisition of the large number of samples needed to determine diagnostic sensitivity and specificity. When presenting the data from this experiment it is important to clearly explain the source and acquisition of samples. Diagnostic samples are not always easy to obtain especially for testing procedures associated with foreign animal diseases. In a validation study, efforts are made to obtain appropriate samples for estimating diagnostic sensitivity and specificity. As populations of animals in which some animals may be infected or diseased are not always available, samples from experimentally challenged animals might be considered to be suitable sample types to validate assays. Experimental challenge studies often include repeated sampling of individual animals to determine the progression of disease, but this is a different objective than estimating the sensitivity and specificity of a test method. Serially drawn samples, taken on different days from the same animal, cannot be used as representative of individual animals in population targeted by the assay, because such samples violate the rule of independence of samples required for such studies. Such samples must be avoided.

Ideally, 60 total samples (30 true positives and 30 true negatives) should be used in this experiment, if possible. Each sample should be from a single animal. Results of the two assays can be compared on true positives (sensitivities) and on true negatives (specificities).

Robotic and manual extraction procedures using diagnostic samples

Robotic and manual extraction procedures were compared by testing positive and negative tonsil, tonsil scraping, and nasal swab samples for African swine fever. Table 4 gives the number of samples that were classified positive by both methods, correlation and concordance correlation which measure repeatability of continuous results (see Barhardt *et al.,* 2007 for details), and kappa estimates for dichotomised results (Maclure & Willett, 1987) with 95% confidence intervals. Examples of and reference to kappa calculations are presented in the accompanying statistical appendix (Appendix 1.1.4.5. Statistical approaches to validation). Figure 7 is an example of the type of graphic display that can be used capture all relevant results.

Table 4. *Correlation and concordance correlation coefficient estimates for each sample type – diagnostic sample comparison example*

Sample Type	***N***	***Correlation***	***Concordance Correlation***	***Kappa***
Tonsils	27	0.71 (95% *CI*: 0.46, 0.86)	0.70 (95% *CI*: 0.44, 0.85)	0.87 (95% *CI*: 0.74, 1.00)
Tonsil scrapings	17	0.74 (95% *CI*: 0.41, 0.90)	0.74 (95% *CI*: 0.42, 0.90)	1.00 (95% *CI*: 0.58, 1.00)
Nasal swabs	9	0.92 (95% *CI*: 0.66, 0.98)	0.80 (95% *CI*: 0.50, 0.93)	0.93 (95% *CI*: 0.80, 1.00)

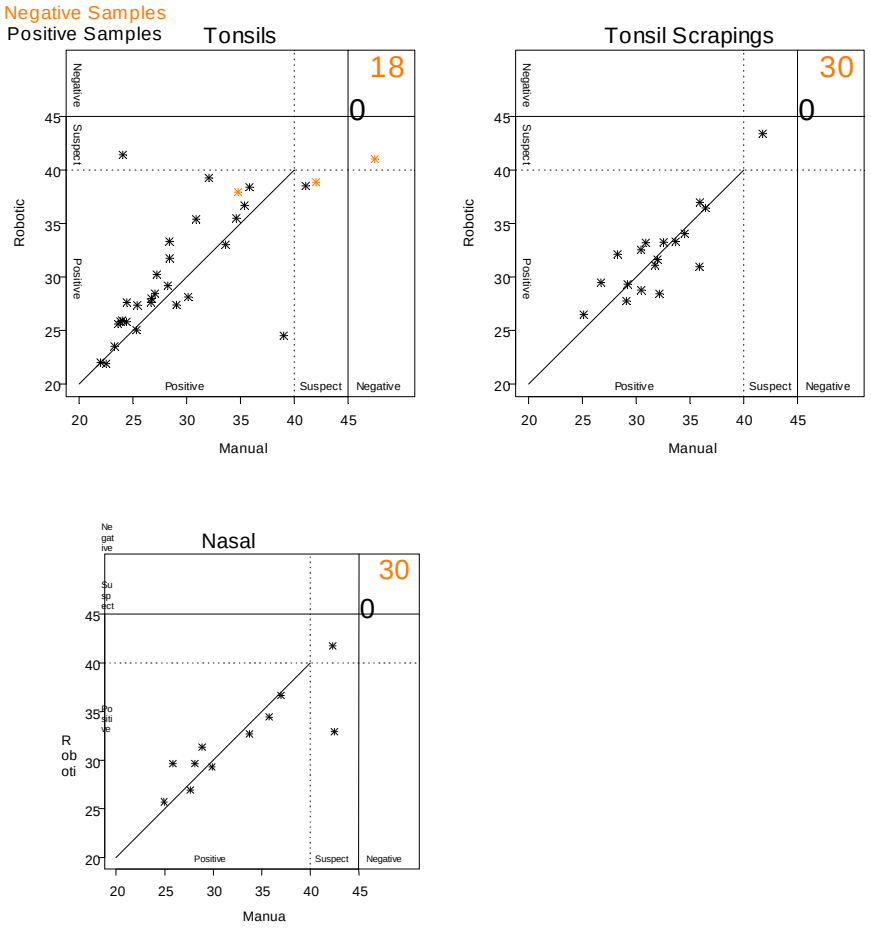


Figure 7. *Sample types data – diagnostic sample comparisons example*

b) Biological modifications to an assay

The above approach can also be taken to compare changes to an assay procedure that may involve the biological reagents used or the specimens to be tested (see Chapter Section B.6.b.ii).

With respect to modifications to the biological reagents used, the choice of diagnostic specimens to run in the original validated method and the substitute method would be as described at the beginning of this

Section. Basically, one well-chosen panel would be run in parallel for the two tests (antibody, antigen or nucleic acid detection) being compared.

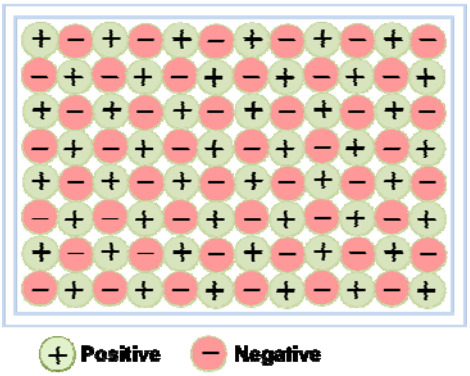
Comparing different hosts/species or different tissues/fluids from a given species requires special consideration and preparation. For example, one may want to compare the testing of a single specimen type in two species using either an antigen or nucleic acid detection system, In this case the species in which the assay had been validated would be the reference species and the other, the candidate species. Target tissues/fluids would need to be collected from 60 individual true-negative animals of each species, matching as best as possible, age, sex, health status, etc. Thirty of these paired specimens would be set aside as negatives for testing purposes. The other 30 pairs, one from each species, should be spiked with an equal amount of the target analyte such that they cover the range of detection in the reference system from high to low. These spiked specimens would be an artificial representation of true positives.

A scenario similar to the one above would involve the comparison of two different target specimens from one species. In this case, the target specimen in which the assay had been validated would be the reference specimen and the other, the candidate specimen. Again, target tissues/fluids would need to be collected from 60 individual true negative animals. As above, 30 of these paired specimens would be set aside as negatives for testing purposes. The other 30 paired specimens (e.g. heart and kidney from individual fish) should be spiked with an equal amount of the target analyte such that they cover the range of detection in the reference system from high to low. These spiked specimens would be an artificial representation of true positives. In this and the above type of comparison, there is an assumption that the distribution of the analyte in different species or different specimens within a species is the same, which of course is not valid. If the analyses demonstrates that the testing of different species or specimens is comparable, all that can be inferred about diagnostic sensitivity is that the performance would be comparable, all things being equal.

5. Assessment of cross contamination

The potential for cross contamination might be an important consideration when equipment or extraction procedures for PCR are changed. Figure 8 shows one possible plate layout for a cross-contamination study. The positive samples should be strong positives. The plate layout shown in Figure 8 alternates the positive and negative samples to maximise the probability of detecting cross contamination. It may be necessary to conduct an experiment to rule out cross contamination for robotic extraction procedures and for some platforms. If the possibility of cross contamination is suspected, this experiment should be conducted prior to any other experiment.

Figure 8. *Example of a cross contamination plate.*



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ADVICE FOR MEMBER COMMENTS

Appendix Number and Title: 1.1.4.7. Selection and use of reference samples and panels

Country making the comments

Date

NB: The Biological Standards Commission is particularly interested in OIE Member Country opinion on this appendix (and its accompanying chapter and six other appendices): are the documents clear and useful? If not, how they could be improved?

It would be appreciated if the following guidance notes are followed in making a reply:

1. *Comments may be general or specific, but specific comments are usually more valuable. General comments should be such that some conclusion and action can be taken in response to them. For example, it is of little help to make statements such as "This test is no longer used in our laboratory", without indicating the reasons and what test is used instead.*

2. *Specific comments should be identified, by indicating the line number in the text, to facilitate the making of editorial changes.*

3. *The pointing out of typing or technical errors is welcome, but the correct word or figure should be indicated. For example, it is of little help to say that 0.8 M is too high, if the preferred value is not indicated.*

4. *Bear in mind that the introductory chapters are not intended to be exhaustive, and indeed none of the chapters can give a completely comprehensive cover of the subject, otherwise the* Terrestrial Manual *would be too long. However, assistance in indicating priorities is always helpful.*

5. *The* Terrestrial Manual *is intended for world-wide use. The chapters need to reflect the development of new technology, but the old established methods, usually requiring less sophisticated apparatus, have to be retained. New technology should not be described in detail until it has gained wide acceptance as a reliable method.*

General Comments

Specific Comments (*add continuation sheets if required*)
line:

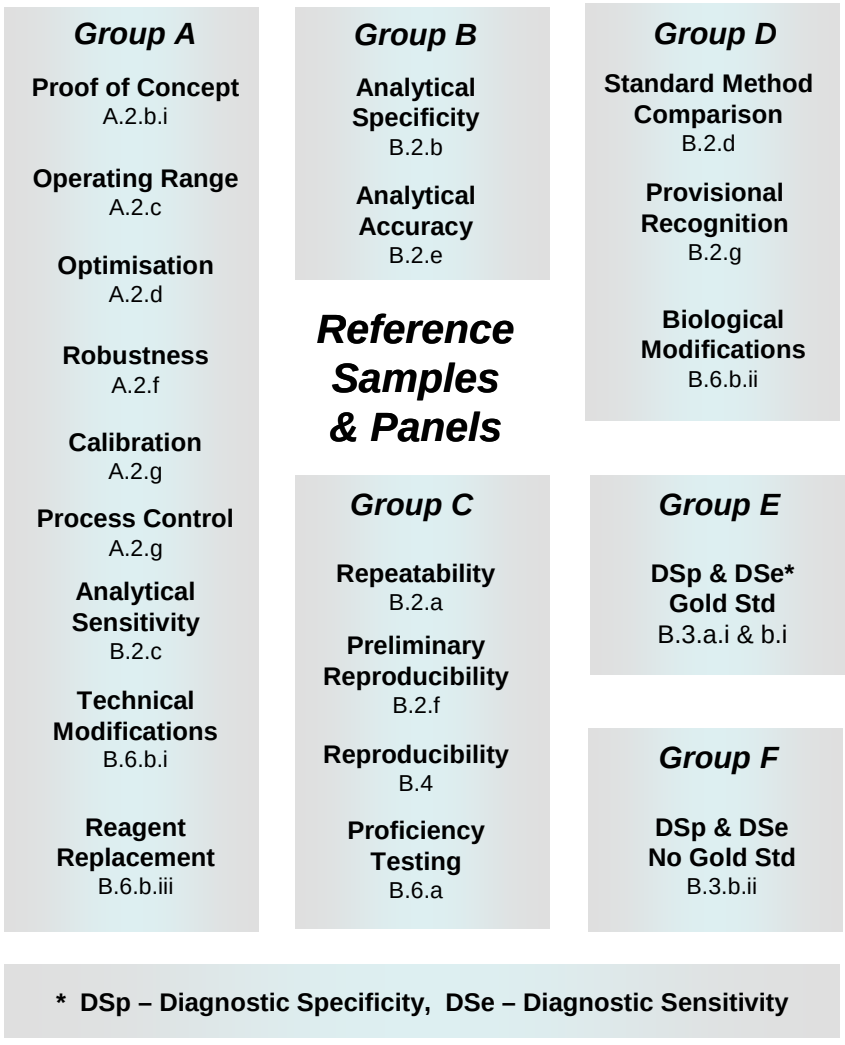
APPENDIX 1.1.4.7.

SELECTION AND USE OF REFERENCE SAMPLES AND PANELS

INTRODUCTION

Reference samples and panels are essential from the initial proof of concept in the development laboratory through to the maintenance and monitoring 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.

Figure 1. *Reference samples & panels grouped based on similar characteristics and composition. The topics and alphanumeric subheadings (e.g. Proof of Concept, A.2.b.i) refer to the relevant section in Chapter 1.1.4/5.*



As can be seen in Figure 1, reference samples and/or panels are mentioned throughout Chapter 1.1.4/5. 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'. In the context of test method validation, reference materials or samples contain the analyte of interest in varying concentrations 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 or excreta that contain the analyte of interest and are usually harvested from infected animals. 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*). *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 Chapter is indicated under each particular application of the reference sample or panel.

1. Group A

The reference samples in this group may be used for multiple purposes from the initial stages of development and optimisation, through Stage 1 and into continuous monitoring and maintenance of the assay. Where possible, large quantities of these reference samples should be collected and/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.

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.

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 to high 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 5 of this Appendix 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 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.

Given all of the above, there is one very important point that needs to be emphasised. No matter whether reference samples are selected from natural sources or prepared in the laboratory, all selection criteria and/or 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.

a) Proof of concept (A.2.b.i)

The OIE *Standard* states that test methods and related procedures must be appropriate for specific diagnostic applications in order for the test results to be of any 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, Chapter 1.1.4/5. 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.

b) Operating range (A.2.c) and analytical sensitivity (A.2.d)

i) Analytical approaches

The operating range of the assay is 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, a high positive reference sample is selected. This high positive sample, natural or prepared, is serially diluted to extinction in a negative matrix representative of sample matrix of samples from animals in the population targeted by the assay. This includes antibody assays where a high positive reference serum should be diluted in a negative reference serum to create the dilution series. Analytical sensitivity is synonymous with the lower limit of detection (LOD) of an analyte in an assay. The same high positive reference sample may be used to determine both the operating range and the analytical LOD. Consult Appendix 1.1.4.6. Comparability of assays after minor changes in a validated test method, for statistical approaches to determining LOD.

ii) Comparative approaches

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 analytical sensitivity 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 analytical sensitivity between the assays, as well as, 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 references 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 section 4. Group D below). Where possible serial samples should be collected from at least five 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 of five per sampling event. For smaller host species, this 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.

c) Optimisation (A.2.d) and robustness (A.2.f)

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 and high 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.

Robustness is a measure of an assay's capacity to remain unaffected by small, but deliberate, variations in method parameters, and provides an indication of its reliability during normal usage. Assessment of robustness essentially begins during the optimisation process. The deliberate variations in method parameters are addressed in experiments after optimal conditions for an assay are established. The same reference samples should be used for both processes, again to provide continuity of evidence.

d) Calibration (A.2.g) and process controls (A.2.g)

i) 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. 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. 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 affect in the candidate assay as there is no matrix control provided. The other approach is to provide a negative and a low and high 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. This compensates for any potentially hidden matrix affect. 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.

ii) 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 both low and high 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.

e) Technical modifications (B.6.b.i)

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 Appendix 1.1.4.6. for statistical approaches to assay precision in the face of technical modifications.

In general, these approaches require the use of three reference samples, a negative and a low and high positive. Again these 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 this enhances the continuity of evidence.

f) Reagent replacement (B.6.b.iii)

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. Whenever possible, 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 this enhances the continuity of evidence and confidence in the assay.

2. Group B

a) Analytical specificity (B.2.b)

By definition, analytical specificity 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 which is often not well understood. Analytical specificity can be further subdivided into i) selectivity, ii) exclusivity and iii) inclusivity. These terms are described in Chapter 1.1.4/5, Section B.2.b.

In the context of assay validation, the choice of reference samples that are required to assess these aspects of analytical specificity is highly dependent on the specific purpose or application that was originally envisaged at the outset development. Assessment of analytical specificity is a crucial element in proof of concept and verification of fitness for purpose.

'Selectivity' refers to 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 test matrix. One aspect of selectivity that is pertinent to the discussion of reference samples relates to tests that are designed to distinguish infected from vaccinated animals, DIVA tests. Reference samples need tobe 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 import that an accurate history be collected with respect to the herds involved, including vaccination practices and disease occurences. Alternatively, it may be necessary to produce this material in experiments like those described in Section1.b.ii of this Appendix, but including a combination of experimentally vaccinated and challenged animals. It is important to avoide use of the vaccine as capture antigen in the assay (e.g. indirect 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. Similar to the comparative approach described above with respect to analytical sensitivity, 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. Depending on the DIVA test, a single experiment could be designed to assess aspects of both analytical sensitivity and specificity.

'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 as 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 candidate assay, these reference materials represent the organism itself, or host-derived samples, or genomic sequences. An exclusivity profile should be established and wherever possible, expanded as potentially cross-reactive organisms arise.

'Inclusivity' is 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 or antibodies thereto. In the initial design of an assay, inclusivity is perhaps one of the most critical design considerations as it is at the crux of the diagnostic question being addressed. In essence, it defines the scope of detection and thus the fitness for purpose. Reference samples are required to define the inclusivity or 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 an inclusivity profile. As with exclusivity, updating these profiles should be an ongoing commitment.

b) Analytical accuracy of adjunct tests (B.2.e)

Some test methods or procedures are solely analytical tools and are usually applied to further characterise an analyte that has been detected in a primary assay, for example assays like virus neutralisation tests used to type an isolated virus or characterise an antibody response. Such adjunct tests must be validated for analytical performance characteristics, but differ from 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. 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, with respect to their source, identity and performance characteristics.

3. 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. 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 1.b.ii of this Appendix. Similar to the comparative approach described above with respect to analytical sensitivity, 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.

a) Repeatability (B.2.a) and Preliminary reproducibility (B.2.f)

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 Appendix 1.1.4.4. 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 and both low and high 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 or more laboratories with a high level 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 Appendix 1.1.4.4. for further explanation of the topic and its application.

b) Reproducibility and Ruggedness (B.4)

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 does the number of variables encountered with respect to laboratory environments, equipment differences and technical expertise. As such, these studies are a measure of an assay's ruggedness, i.e. the 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, within-laboratory repeatability estimates may be augmented by replicate testing of these samples when used in the reproducibility studies.

c) Proficiency testing (B.6.a)

A validated assay in routine use in multiple laboratories needs to be continuously 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, semi-quantitative results provide additional data for assessment of non-random error among the participating laboratories.

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 Appendix 1.1.4.4. for further explanation of the topic and its application.

4. 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 Appendix 1.1.4.6., experimental challenge studies often include repeated sampling of individual animals to determine the progression of disease, but this is a different objective than comparing performance characteristics that would be associated with diagnostic sensitivity and specificity of a test method. Serially drawn samples, taken on different days from the same animal, cannot be used as representative of individual animals in population 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.

a) Standard method comparison (B.2.d) and Provisional recognition (B.2.g)

There are situations where it is not feasible or desirable to continue to 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 on the candidate assay method and by the standard method in current use. A standard test method is the best internationally or nationally recognised method, or in their absence, the best available method preferably published in a reputable journal. If the methods are deemed to be comparable (Appendix 1.1.4.6.), 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.

As above, experience has shown that the greatest obstacle for 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 (Chapter 1.1.4/5., Section B.3). 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

Chapter. 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 to a standard method or provisional recognition, a panel of 60 samples should be assembled. This would include 30 'true' negatives and 30 'true' positives. Where 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 Appendix 1.1.4.6. for statistical approaches to determining methods comparability using diagnostic samples.

b) Biological modifications (B.6.b.ii)

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 Appendix 1.1.4.6. for statistical approaches to determining methods comparability using diagnostic samples.

5. Group E

Reference animals and reference samples in this Group E are well described in the Chapter 1.1.4/5., Sections B.3.a–c). However, there are a few points that are worth re-iterating here.

a) Gold standard [1] – diagnostic specificity (B.3.a.i) and diagnostic sensitivity (B.3.b.i)

For conventional estimates of diagnostic specificity, 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 has either been eradicated or has never had the disease in question.

Again for conventional estimates of diagnostic sensitivity, positive reference samples refer to true positives. 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 Chapter 1.1.4/5., Section B.3.i.

The important point here is that all samples, irrespective of origin, must be documented as they would for any other reference sample. As mentioned in Section 1 of this Appendix, 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 clinical signs, antibody profiles, pathogen load or shedding, etc. In some cases, experimental infection/exposure may be the only viable option for the production of reference material (see Section B.3.c) In this case, all of the above plus the experimental protocol should be detailed.

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 Appendix 1.1.4.5. Statistical approaches to validation for statistical considerations.

1 The term "Gold Standard" is limited to a perfect reference standard as described in Chapter 1.1.4, Section B.3.b.i, and Appendix 1.1.4.5., Introduction and Figure 1.

6. Group F

a) No gold standard – diagnostic specificity and diagnostic sensitivity (B.3.b.ii)

Latent-class models are introduced in Chapter 1.1.4/5. 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 Appendix 1.1.4.5. for statistical considerations.

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

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* *

ADVICE FOR MEMBER COMMENTS

Chapter Number and Title: 1.1.7. Application of biotechnology to the development of new and emerging diagnostic technologies

Country making the comments

Date

It would be appreciated if the following guidance notes are followed in making a reply:

1. *Comments may be general or specific, but specific comments are usually more valuable. General comments should be such that some conclusion and action can be taken in response to them. For example, it is of little help to make statements such as "This test is no longer used in our laboratory", without indicating the reasons and what test is used instead.*

2. *Specific comments should be identified, by indicating the line number in the text, to facilitate the making of editorial changes.*

3. *The pointing out of typing or technical errors is welcome, but the correct word or figure should be indicated. For example, it is of little help to say that 0.8 M is too high, if the preferred value is not indicated.*

4. *Bear in mind that the introductory chapters are not intended to be exhaustive, and indeed none of the chapters can give a completely comprehensive cover of the subject, otherwise the* Terrestrial Manual *would be too long. However, assistance in indicating priorities is always helpful.*

5. *The* Terrestrial Manual *is intended for world-wide use. The chapters need to reflect the development of new technology, but the old established methods, usually requiring less sophisticated apparatus, have to be retained. New technology should not be described in detail until it has gained wide acceptance as a reliable method.*

General Comments

Specific Comments (*add continuation sheets if required*)
line:

This chapter has been extensively revised and updated. Although some portions of the exisiting text have been incorporated, new text and deleted text have not been marked, in the interests of clarity.

CHAPTER 1.1.7.

THE APPLICATION OF BIOTECHNOLOGY TO THE DEVELOPMENT OF NEW AND EMERGING DIAGNOSTIC TECHNOLOGIES

INTRODUCTION

Modern concepts of livestock breeding and increased global trade demand the development of early warning diagnostic platforms, global capacity and diagnostic harmonisation to enhance livestock production and health and contribute towards uniting pathogen detection technologies as a basis of the global one-world, one-health initiative.

The global development of improved transport infrastructure has contributed to increased international movement of people and goods. Consequently, the risk of pathogen transfer between countries or even between continents is increased. In response to global climate changes, diseases endemic in certain areas, such as bluetongue and African swine fever, are showing a tendency to move or extend to other geographical areas outside their normal range. Finally, the constant intensification of animal production requires different approaches related to the preventive measures, diagnosis and control of animal diseases.

In order to be widely accepted, modern diagnostic methods have to be sensitive, specific, rapid, user friendly, cost effective, and eventually automated to facilitate the evaluation of large numbers of samples.

Eradication of rinderpest is a unique example of the application of new technologies to the control and eradication of a disease, where the virus neutralisation test was replaced with a well validated enzyme linked immunosorbent assay that was used in the eradication programme worldwide.

The purpose of this chapter is to provide general background information for the non-specialist. Two issues of the OIE Scientific and Technical Review *are concerned with biotechnology and the diagnosis of animal diseases and are available on the OIE website. The following is an outline of the topics briefly reviewed in this chapter.*

A. Detection of nucleic acids

1. Nucleic acid extraction
2. Thermal and isothermal polymerase chain reaction (PCR) and real-time PCR
   a) Thermal PCR principle
   b) Isothermal PCR principle
3. Detection of nucleic acids without DNA amplification
4. Diagnosis by restriction fragment length polymorphisms and related DNA-based approaches
5. Genome sequencing
6. Diagnosis by DNA probes and DNA microarray technology

B. Detection of proteins

B1. Methods for detection of antigens and antibodies

1. Agglutination
2. Haemaglutination inhibition test
3. Agar gel immunodiffusion test
4. Immunoblotting

B2. Detection of antigens

1. Antigen-capture enzyme-linked immunosorbent assay (*AgELISA*)
2. Quantitative immunoassays (*radioimmunoassay and quantitative enzyme immunoassay*)
3. Immunohistochemistry
4. Immunochromatography
5. Proteomics

B3. Detection of antibody

1. Complement fixation test
2. Indirect ELISA (*iELISA*)
3. Competitive ELISA (*cELISA*)
4. Blocking ELISA (*bELISA*)
5. Production of antigens by recombinant DNA technology
6. Developing technologies for protein detection

B4. Implications of the new technologies

A. DETECTION OF NUCLEIC ACIDS

1. Nucleic acid extraction

An important step in the molecular diagnostic procedure is extraction of 'clean' nucleic acid mixtures to act as a template for the reactions. While it is relatively easy to extract DNA from bacterial cultures or blood, it is technically more challenging to prepare suitable material from field samples, such as faeces, internal organs or abortion material. If the target material has not been purified of contaminants in the clinical sample, the assay is compromised and may yield false results. Several types of samples are known to inhibit the polymerase chain reaction (PCR) reaction, leading to false-negative results. On the other hand, having to process large numbers of samples increases the risk of contamination, leading to false-positive results. There are a number of specialised methods for particular types of samples and tissues, most of which are now commercially available either as manual or automated systems for robotic workstations. The development and accessibility of the robotic extraction platforms not only minimises the risk of contamination, but also enables processing of large numbers of samples under constant reaction conditions and minimal operator manipulation. Consequently, these platforms have contributed to the establishment of high-throughput, robust diagnostic assays, shortening the processing time required per sample from hours to minutes (Belák *et al.,* 2009). These are destined to improve the reliability of nucleic acid extraction from different samples, but it still remains a challenging area.

As an alternative to nucleic acid extraction, biotechnologists are increasingly focusing on polymerases that are resistant to PCR inhibitors and several are now available on the market for direct amplification of nucleic acids from pathological specimens without any extraction step.

2. Thermal and isothermal polymerase chain reaction (PCR) and real-time PCR

The PCR exploits natural DNA replication mechanisms and results in the *in-vitro* production of large quantities of a desired sequence of DNA from a complex mixture of heterogeneous sequences (Saiki *et al.,* 1988). PCR can amplify a selected region of from fifty to several thousand base pairs into billions of copies. The specificity of the amplified region can be targeted by specific primers (short synthetic molecules of DNA complementary to both strands and flanking the target sequences), which are annealed to the single-stranded template and extended with the DNA polymerase.

The identity of the PCR product is defined by its characteristic size, and/or confirmed using DNA probes (see below), or restriction digests, which can be used to provide restriction fragment length polymorphisms (RFLP) (see Section A.4). More commonly, since the advent of automated cycle sequencing techniques, identification can be made unequivocally via direct sequencing of the PCR product. For example, sequencing has been used in the virulence typing of avian influenza A virus, in which virulence-associated structural motifs at the haemaglutinin gene cleavage site are reliable indicators of high pathogenicity in chickens (Horimoto & Kawaoka, 1995). The sensitivity of a PCR may be enhanced by the use of a second set of primers to amplify a sub-fragment from the PCR product of the first reaction. This technique is commonly referred to as 'nested PCR' and has been used to detect low levels of pathogens in the sample. However, the use of nested PCR can increase the rate of environmental contamination and consequently increase the risk of false-positive results.

PCR is a highly sensitive procedure for detecting infectious agents in host tissues and vectors, even when only a small number of host cells are infected. PCR can target and amplify a gene sequence that has become integrated into the DNA of infected host cells. It can also target and amplify unintegrated viral gene sequences. It is clear that PCR has a role in the testing of vaccines to detect contamination. However, it does not differentiate between viable and nonviable organisms or incomplete pieces of genomic DNA, and this may complicate interpretation of results and affect the applicability of PCR in this role.

PCR may prove to be very useful in the diagnosis of chronic, persistent infections, such as bovine viral diarrhoea (BVD), enzootic bovine leukosis or caprine arthritis/encephalitis virus. These diseases present serious problems in terms of diagnosis and prevention as infected animals are a constant potential source of transmission.

To expand its utility in veterinary diagnostics and pathogen identification, PCR has been extensively modified over the years. PCR using broadly conserved primers is designed for identification of classes of pathogens. Using PCR primers that are complementary to these conserved sequence regions, the presence in the sample of any bacteria of a desired class can be determined. It must be noted that a positive PCR result needs to be further characterised by hybridisation with species-specific probes, analysis by RFLP, or by sequencing. Similarly, consensus PCR can be designed to use degenerate primers conserved sequence regions or motifs of a group of related pathogens. The targeting of degenerate primers has led to the identification of many previously unrecognised viruses in various animal species. On the other hand, multiplex PCR has been designed to use two or more primer pairs directed at pathogen-specific unique sequences within a single reaction for simultaneous detection of multiple pathogens. Multiplex PCR has the advantage of a high degree of sensitivity and specificity. However, there have been reports that multiplexing can reduce sensitivity compared with single reactions, because of competition. If it is important to have a very sensitive assay, this should be considered.

Classical PCR methods for diagnosis of pathogens, both bacterial and viral, are now widely replaced with real-time PCR assays. In real-time PCR assays, intercalating dyes or a target-specific probe or primer (labelled with fluorescent dye) are used. The measured fluorescent signal is proportional to the number of specific DNA fragments produced. Thus, during the real-time PCR, the accumulation of PCR products can be monitored in each consecutive cycle as a change in the degree of fluorescence. In other words, the assay can be used for absolute or relative quantification of the DNA or RNA content in a given sample. In contrast to conventional PCR, real-time PCR requires less manipulation, is more rapid, has a closed-tube format (decreased risk of cross-contamination), is highly sensitive and specific thus retaining qualitative efficiency, and provides quantitative information. In many cases, real-time PCR assays have proved to be more sensitive than existing reference methods. The development of portable real-time PCR machines and assays raises the prospect of these techniques being used for rapid (less than 2 hours) diagnosis of disease outbreaks in the field.

a) Thermal PCR principle

The amplification of DNA in thermal PCR protocols is accomplished using a succession of cyclic incubation steps at different temperatures. The target DNA is first heat-denatured to separate the two complementary strands to provide a single-stranded template. Specific primers are then annealed to the single-stranded template at a low temperature and extended with DNA polymerase at an intermediate temperature. Once the polymerase has synthesised a new strand of DNA, the product is separated from the template by heating to a higher temperature. These steps, referred to as cycles, are repeated 20–40 times, resulting in amplification of target DNA sequences. The key to the geometric amplification of target DNA sequences by the PCR is the selection of paired primers that, when extended, will create additional reciprocal primer-annealing sites for primer extension in subsequent cycles. To detect RNA (e.g. RNA viruses), a cDNA copy of the RNA must first be made using reverse transcriptase (RT). The cDNA then acts as the template for amplification by the PCR. This technique is referred to as reverse transcription PCR (RT-PCR).

b) Isothermal PCR principle

Isothermal amplification PCR technologies offer the advantage of omitting thermocycling, enabling DNA amplification at constant temperature. These technologies include nucleic acid sequence-based amplification (NASBA), transcription-mediated amplification (TMA), signal-mediated amplification of RNA technology (SMART), strand displacement amplification (SDA), rolling circle amplification (RCA), loop-mediated isothermal amplification (LAMP), isothermal multiple displacement amplification (IMDA), Helicase-dependent amplification (HDA), circular helicase-dependent amplification (cHDA), single primer isothermal amplification (SPIA) and strand invasion-based amplification (SIBA) (Gill & Ghaemi, 2008). The most widely used method is the LAMP PCR, which deploys four primers forming a stem-loop DNA by self-primed DNA synthesis and a DNA polymerase with strand displacement activity. The result of the amplification process is the production of loops at the ends of the complementary strands, which are continually extended. The amplification process is indicated by either the occurrence of turbidity in the reaction mixture or the generation of a fluorescent signal using fluorescent dyes (Gill & Ghaemi, 2008).

When PCR is used for diagnosis, a great deal of care is required to avoid contamination of the samples because the exquisite sensitivity of the technique can easily lead to false-positive results. Multicentre studies

have shown that positive samples are detected consistently between laboratories, but that false positives are frequently obtained with known negative samples, indicating the continuing presence of contamination problems (Schweiger *et al.,* 1997). A new generation of robotic workstations is now available where PCR reactions may be set up with only a single tube open at any one time. This greatly reduces the risk of contamination. It is also important to control for potential 'negative' results caused by the presence of PCR inhibitors in the reaction mixture. A template, independent of the target DNA, known to produce a PCR product (mimics) with specific primers can be used as a control for the PCR inhibitors, thus indicating false-negative results (Belák *et al.,* 2009). Use of these precautions allows the PCR to become a realistic option for the diagnostician.

The generation of the signal in a real-time PCR assay has been limited, until recently, to certain chemistries, such as intercalating dyes (SYBR Green and EvaGreen), hydrolysis probes, molecular beacons, primer probe energy transfer (PriProET), scorpion primers, dual hybridisation probes and dye-labelled oligonucleotide ligation (Belák *et al.,* 2009). Alternative labelling has been developed using tags that enable high multiplexing of the assay. The mass tag PCR assay is an improvement of the real-time PCR platform, in which the primers are tagged with tags of known, but different, molecular weights. After amplification of the targeted DNA fragments, the tags are released using UV light and subsequently measured using mass spectrometry. This approach enables multiplexing of much larger panels of target DNA fragments (and hence multiple diseases), as the assay is not limited to the number of dyes available. Application of the mass tag PCR assay has been already proven in differential diagnosis of syndromic diseases (respiratory, haemorrhagic, enteric pathogens, meningitis/encephalitis syndrome) and detection of new clades of pathogens (Dominguez, 2008; Lipkin, 2010). A modification of this method uses matrix-assisted laser desorption-Ionisation (MALDI), which directly measures the molecular weights of the PCR products and compares them with known databases (Lipkin, 2010).

3. Detection of nucleic acids without DNA amplification

A new approach to detection of target DNA has been developed using surface-enhanced Raman scattering (SERS) (Harpster *et al.,* 2009). The method is based on capturing a thiol-conjugated probe and a methylene blue-labelled reporter probe, which are attached to the complementary (target) DNA. A Raman label positions the methylene blue on a gold nanoparticle, to an optimal distance for SERS enhancement. The production of a measurable, capture-specific signal of the Raman spectrum is produced by elicitation of surface-enhanced plasmon resonance by laser excitation. The analytical sensitivity of the method is in the nanomolar range. The signal can also be measured by quartz crystal microbalance-dissipation (a method based on the induction of a piezoelectric effect), which enables quantitative interpretation of the results. A modification of this method uses paramagnetic nanoparticles as a carrier for the capture probe. Once the target DNA is attached to the capture and reporter probes, the complexes are attracted magnetically to a defined position, where the "concentrated" signal is measured. Quantitative evaluation of the results is also possible using this method.

4. Diagnosis by restriction fragment length polymorphisms (RFLP) and related DNA-based approaches

Serological tests that are commonly used to identify microorganisms may not distinguish between isolates of closely related pathogens, whether they are viruses, bacteria, fungi or parasites. A DNA-based procedure will offer the higher degree of discrimination that is often required and an appropriate starting point may be analyses for restriction fragment length polymorphisms (RFLP).

The RFLP approach is based on the fact that the genomes of even closely related pathogens are defined by variation in sequences. For example, the linear order of adjacent nucleotides comprising the recognition sequence of a specific restriction enzyme in one genome may be absent in the genome of a closely related strain or isolate.

In practice the RFLP procedure consists of isolating the target pathogen, extracting DNA or RNA (with subsequent reverse transcription to cDNA), and then digesting the nucleic acid with one or a panel of restriction enzymes. The individual fragments within the digested DNA are then separated by gel electrophoresis and visualised by staining with ethidium bromide. Ideally each strain will reveal a unique pattern, or fingerprint. Many different restriction enzymes may be considered at the outset, so that analyses of many molecular fingerprints from digestions with several individual restriction enzymes may be undertaken; a combination of the best set of results will allow a comprehensive differentiation between strains or isolates (Loza-Rubio *et al.,* 1999).

Of greater utility to the study of pathogens is a modification to the basic RFLP technique whereby the PCR is incorporated as a preliminary step. The PCR method is used to amplify a specific region of the genome (known already by the investigator to be variable in sequence between different pathogens), which then serves as the template DNA for the RFLP technique. This combination (PCR-RFLP) offers a much greater sensitivity and comparability for the identification of pathogens and is especially useful when the pathogen is present in the infected animal in low numbers or is difficult to isolate in culture. The involvement of specific strains or types in a

disease outbreak can be thus determined and the epidemiological tracing of isolates within a country or between countries should be possible.

The incorporation of pulsed-field gel electrophoresis (PFGE) facilitates the separation of large (up to megabase size) fragments of DNA and can be a useful adjunct to the basic RFLP analysis (Jager *et al.,* 1998).

RFLPs have a clear value for use in epidemiological studies but more critical interpretation of RFLP data involves the construction of databases to determine whether the RFLP profiles are clinically significant and linked to factors such as virulence or host range. In practice, it is usual not to rely on one restriction site but to use sites from several locations within the genome to classify an isolate. A continuing dilemma for veterinary diagnosticians is the correct assessment of any molecular differences that might be found between different isolates of a pathogen, as the loss or acquisition of restriction endonuclease site(s) may not be associated with differences in the ability of the pathogen to cause disease, i.e. an RFLP difference may not be functionally significant, except as a distinguishing feature.

Polymorphic random amplified polymorphic DNA (RAPD) markers that define individual strains, etc., may be sequenced and then used as a sequence-confirmed amplified region (SCAR). Thus conversion of an anonymous polymorphic marker to a SCAR means that a single PCR may be done to more simply identify a specific genome (Lewin *et al.,* 2002).

Although the RFLP and PCR-RFLP are much less powerful compared with the modern sequencing technologies, they are inexpensive, easy to perform and sufficiently descriptive for epidemiological investigations of outbreaks and identification of individual strains of pathogens. The RFLP/PCR-RFLP motif investigation is a valuable tool to differentiate the origin of an infection (especially where animal-to-human transfer has occurred) (Laroucau *et al.,* 2009) and/or to differentiate between field and vaccinal strains of individual pathogens (Laroucau *et al.,* 2010).

5. Genome sequencing

The techniques by which DNA from a pathogen may be detected and characterised continue to improve and evolve. Presently, the ultimate discriminatory procedure is that of genome sequencing.

Conventional genome sequencing is based on PCR amplification of targeted DNA fragments with labelled di-deoxy nucleotides, which have a property to stop the elongation at their place of binding. Each di-deoxy nucleotide is labelled with different dye, enabling distinction between individual di-deoxy nucleotides. As each di-deoxy nucleotide competes with the 'normal' nucleotides for their complementary binding sites, the result of such PCR amplification will be a mixture of DNA fragments of different length, each ending with a defined di-deoxy nucleotide (identifiable by its colour). The PCR mixture is than analysed using capillary electrophoresis, which separates the fragments by length and reads the colour of each fragment. Analytical software is then used to convert the colour signals to a layout of nucleotides.

Since 1977, the Sanger method (Sanger *et al.,* 1977) has been the dominant approach and gold standard for DNA sequencing. The commercial launch of the first massively parallel pyrosequencing, 454 DNA sequencer platform in 2005, ushered in the new era of high-throughput genomic analysis now referred to as next-generation sequencing (NGS). This allows the sequencing of a large genome in a short time, facilitating the study of genetic material recovered directly from environmental samples, or metagenomics. These new technologies have made it possible quickly to identify an unknown pathogen (emerging pathogens) or one difficult to cultivate *in vitro,* or to identify a variant that is present in small quantities within a mixture (Kunin *et al.,* 2008).

Development of microarray technologies, as well as the improvements in DNA manipulation, has contributed significantly to the development of direct sequencing protocols capable of detecting unknown pathogens. This technology enables sequencing of large DNA fragments, allowing sequence comparison with sequence databases available locally or in the public domain.

Sequencing of a well characterised portion of the genome is playing an important role in pathogen characterisation and epidemiological studies. Sequencing the products amplified by PCR using degenerate primers targeting a gene common to the viruses in the same family has become an important diagnostic tool, especially for identification of previously unrecognised members of the family. Sequencing the products in a defined region of the genome is used in epidemiological studies to evaluate the genetic similarity to other pathogens of the same species (subtype), to determine the phylogenetic properties or to determine the origin of an outbreak/infection. Additionally, by analysing and comparison of different sequence motifs, this technology offers the possibility of predicting the tendency of pathogens to mutate into more pathogenic strains, allowing, to a certain level, tracing forward the spread of an outbreak or infection (Horimoto & Kawaoka, 1995).

The state of the art technologies, such as high mass tag-PCR and high-throughput sequencing (Belák *et al.,* 2009; Lipkin, 2010) have opened much greater diagnostic opportunities, compared with conventional targeted

sequencing. The mass tag-PCR has been successfully used for simultaneous detection and differentiation of syndromic diseases, such as respiratory diseases, diarrhoeas, encephalitides/meningitides and haemorrhagic fevers (Lipkin, 2010). Moreover, high-throughput sequencing applied in multiplex platforms is capable of generating random whole genome sequencing, giving the opportunity for simultaneous pathogen detection and comparison in different regions of the genome.

Development of high-throughput sequencing has created a demand for a different approach to data management because of possible amplifications of high numbers of unexpected pathogens and their interactions with the host cell genome (Kunin *et al.,* 2008). The process of differentiation of these pathogens, when performed using conventional blasting, is still time-consuming and inappropriate for routine use. Several approaches are currently under development to solve this problem, such as sample preparation (removal of the host cell eukaryotic DNA during the extraction phase), reducing the entry datasets for evaluation (submitting parts of the obtained amplicons instead of the whole genome), targeting towards a limited (reduced) panel of pathogens (e.g. animal pathogens, only viruses/bacteria, only a group of viruses/bacteria, etc.) and optimisation of bioinformatics (production of specialised software platforms capable of analysing large amounts of data using built in algorithms). Each of these approaches has advantages and disadvantages, however it seems that careful sample preparation and optimisation of bioinformatics can offer the best solutions.

6. Diagnosis by DNA probes and DNA microarray technology

Conventional DNA probing and microarray analysis are different but closely related processes. Fundamental to both processes is the binding (hybridisation) of DNA, derived from a sample suspected of containing a pathogen (the 'unknown'), with highly characterised DNA derived in advance from a pathogen of interest (the 'known' DNA).

In conventional DNA probing, the unknown DNA (or RNA) – the target – is immobilised on a solid surface e.g. a filter. The known DNA, made into a probe by labelling or tagging it in some way, is in the liquid phase and is applied to the target. In microarray diagnosis, it is the known DNA (large oligonucleotides or complementary DNA) that is the target, immobilised on a glass slide, and the unknown DNA, in the liquid phase, that is labelled to make a probe.

In conventional DNA probing, the target can be nucleic acids extracted from clinical material or cultured cells and either (a) added to filters (a dot or slot blot) or (b), less conveniently in a diagnostic context, transferred to a filter after gel electrophoresis. The amount of pathogen in a clinical sample might be too low for detection. Consequently the nucleic acid might be amplified by PCR or RT-PCR, the PCR product being applied to a filter. To visualise a probe bound to its target, the probe can be labelled with a radioactive nuclide or, more commonly and safely, 'tagged' non-radioactively. For example, biotin or psoralen–biotin may be incorporated into the probe, the bound probe then being detected by addition of streptavidin linked to an enzyme for subsequent generation of colour or light (chemiluminescence).

A microarray is so-called because it can comprise several thousand different known DNAs, each DNA being spotted onto glass slides to form the array. Each spot is only around 10 µm in diameter. DNAs complementary to parts of selected genes of pathogens can be used to make the arrays (Belák *et al.,* 2009). However, if large numbers of pathogens are to be investigated then it would be easier logistically to use large oligonucleotides. In microarray probing the probe is made from the nucleic acid of the test sample. The nucleic acid is extracted from a sample and a PCR or RT-PCR performed using random oligonucleotide primers. In this way, part of all the nucleic acids in the sample – both of host and pathogen origin – are amplified. These PCR products, representative of every nucleic acid in the sample, are labelled with a fluorescent dye and applied to the microarray. Under optimised conditions only the DNA derived from the pathogen will bind to the DNA on the glass slide. If detection of a particular pathogen or group of related pathogens is the object then pathogen-specific oligonucleotides can be used to amplify these within the sample for probe production.

Microarrays for detecting pathogens can be designed for several levels of differentiation. In the case of oligonucleotide target DNAs oligonucleotides may initially be designed to be able to detect and differentiate pathogens at the genus level. A number may be chosen, perhaps 10 or so, of oligonucleotides with a high degree of sequence conservation (consensus oligonucleotides) within a given genus, such that a probe made from a field sample containing a member of that genus would be likely to hybridise to at least some of the oligonucleotides, while not hybridising (or hybridising to a lesser degree) to those corresponding to related genera, e.g. to differentiate Apthovirus (foot and mouth disease virus [FMDV]) isolates from Enterovirus strains in the Picornaviridae family. Other sets of oligonucleotides, placed on the same array slide, able to characterise a pathogen more specifically, e.g. to differentiate the seven types of FMDV, and potentially to even further refinement at the subtype level, could then be selected.

In conventional DNA probing the detection of a pathogen is limited by the number of probes used, whereas microarray analysis is limited only by the number of target DNAs on the array. If a microarray has 1000 different oligonucleotides, then to achieve the same resolving power by conventional probing would require 1000 probes and 1000 separate probing reactions. The great advantage of microarray analysis in searching for pathogens is

that hundreds of pathogens can be looked for simultaneously when probing a single microarray slide. Clearly, microarray analysis has great potential when investigating diseases of unknown aetiology or diseases where more than one pathogen might be present and when subtyping is required. To enhance sensitivity in pathogen detection, microarrays can be coupled with PCR amplifications. These PCRs are usually designed to amplify one or more conserved genes, or multiple sequences, such as PCR using broadly conserved primers, consensus PCR and multiplex PCR. When a particular pathogen needs to be identified, then the use of a microarray would be less justifiable, as the production and hybridisation of slides is relatively expensive. Instead, for these more simple cases, it would be more appropriate to use pathogen/subtype specific PCRs, followed by sequencing or RFLP for confirmation.

Currently, most of the novel technologies, such as mass tag PCR, SERS, high-throughput sequencing, etc. are compatible with certain microarray formats. This enables high multiplexing of the tests in a single run, enabling generation of a large amount of data from a single sample. Appropriate software are used for internal quality control/quality assurance purposes in the microarrays, as well as for data recording related to the status of an individual sample.

If past developments in biotechnology are indicative of the future, then microarray equipment and reagents would be expected to become less expensive, leading to greater application of this technology in animal disease diagnosis. It will assist in the search for hitherto undiscovered viruses or the characterisation of bacterial strains in terms of virulence, anti-microbial sensitivity or other important markers. One of the main challenges faced when using array-based approaches is the handling and analysis of the very large data sets that are generated.

B. DETECTION OF PROTEINS

B1. METHODS FOR DETECTION OF ANTIGENS AND ANTIBODIES

1. Agglutination

Agglutination is a method using the property of specific antibodies to bind many pathogenic organisms into single clumps thereby forming large complexes, which are easily precipitated. The precipitation can be macroscopically or microscopically visible. Different technical applications of this principle are still widely used in veterinary laboratories, especially for detection of specific antibodies against brucellosis and leptospirosis (see disease specific chapters in this *Manual*). The method can be used for detection of both antibodies, when known antigen is used, or antigen, when well defined sera are used (European Commission [2001]).

2. Hemaglutinition inhibition test

The haemagglutination inhibition method relies on the property of some pathogens (mainly viruses) to nonspecifically agglutinate erythrocytes. In the presence of specific antibodies, this actvitiy of the virus can be ‘blocked’ and it will be inhibited from agglutinating the erythrocytes. The test is used for qualitative and quantitative detection of both antigens and antibodies. It has been used extensively for detection of serotype-specific antibodies against avian influenza, peste des petits ruminants (PPR) and others. The test can be also used for detection of antigen (detecting presence of an avian influenza virus from the allantoic fluid after attempted isolation) (Mulder & Brans, 1952).

3. Agar gel immunodiffusion test

The agar gel immunodifussion test is based on the property of proteins to randomly diffuse in an agar of certain concentration. Antibodies and antigen are placed in wells at a defined distance. After incubation, at the site in the agar gel where antibodies and the antigen meet and bind, precipitation lines are formed, visible under a beam of intense oblique light against a black background. The test is most commonly used for detection of antibodies against avian influenza (matrix antibodies), equine infectious anaemia and enzootic bovine leukosis. Under certain circumstances the test can be used for detection of antigen (monitoring the success of avian influenza virus isolation in allantoic fluid) (Coggins *et al.,* 1972).

4. Immunoblotting

Immunoblotting combines the high resolution of gel electrophoresis with the specificity of immunochemical detection and offers a means of identifying immunodominant proteins recognised by antibodies from infected animals or monoclonal antibodies (MAbs) directed against the target agent. The immunoblotting procedure can be divided into six steps: (i) Preparation of the sample, (ii) Resolution of the antigen by gel electrophoresis, (iii) Transfer of the separated polypeptides to a membrane support (nitro-cellulose membrane), (iv) Blocking nonspecific binding sites on the membrane, (v) Incubation with detecting antibody, and (vi) Detection of bound antibody.

The choice of detecting antibody is critical. Polyclonal sera are composed of a range of antibodies reflecting the full repertoire of the immune response to a particular complex antigen. They will therefore detect a number of distinct polypeptides giving a characteristic 'profile' of reactivity. MAbs bind to only one epitope; therefore they are useful in identifying highly specific polypeptides. After incubation with the detecting antibody, any antibodies bound to specific protein bands are visualised using enzyme-labelled anti-species antisera and a suitable substrate/chromogen.

Immunoblotting is performed chiefly in diagnostic laboratories to identify and/or characterise infectious agents based on antigen specificity or to use of known antigens to detect a specific serological response. False-positive and false-negative results in other diagnostic assays can often be resolved by immunoblotting (Molina Caballero *et al.,* 1993). As an example of antigen detection, immunoblotting is a major screening method for bovine spongiform encephalopathy (BSE) and scrapie; it has been used on millions of brain stem samples in Europe and elsewhere for the detection of prion protein (Schaller *et al.,* 1999). Immunoblotting is also often used to determine the specificity of individual MAbs. Individual purified polypeptides (or recombinant expressed proteins) may also be transferred to nitrocellulose membranes by immunoblotting to examine the reactivity of test sera to individual proteins. This characteristic profile of reactivity may be used to help distinguish between animals that have been vaccinated or infected, such as the enzyme-linked immunoelectrotransfer blot (EITB), a western blot for FMD that is widely used in South America (Bergmann *et al.,* 1993). The major factor affecting the success of an immunoblotting technique is the nature of the epitopes recognised by the antibodies. Most high resolution gel techniques involve some form of denaturation of the antigen. This destroys conformational determinants and allows only the detection of linear or non-conformational epitopes. Most polyclonal antisera contain antibodies to both linear and conformational epitopes, but MAbs are directed at single epitopes; thus if they target conformational epitopes, they will not react with denatured protein.

B2. DETECTION OF ANTIGENS

1. Antigen-capture enzyme-linked immunosorbent assay (AgELISA)

The antigen-capture enzyme-linked immunosorbent assay (AgELISA) facilitates detection of antigen from pathogens present in an animal prior to or during clinical disease. The AgELISA commonly follows a sandwich assay format using capture and detecting antibodies (either specific MAbs or polyclonal antibodies). Antigen from the test sample is first captured by a specific MAb or polyclonal antibody bound to a solid-phase support and its presence is detected through use of a second MAb or polyclonal antibody, which may either be radio- or more generally, enzyme-labelled. If the detecting antibody is not labelled then an anti-species conjugate (reactive to the detector antibody) is used. The capture antibody selects the target antigen from other competing protein in sample suspensions and ensures that it is semi-concentrated to increase the chances of its detection. The desired characteristics of the capture MAb are strong binding to the pathogen, recognition of a conserved epitope highly specific for the target agent, and the ability to attach to an ELISA plate without loss of reactivity. In addition, a second MAb recognising an epitope other than that recognised by the capture MAb that is bound to the ELISA plate is often used as part of the indicator system. However, it may be difficult to identify MAbs of comprehensive intra-type reactivity and polyclonal antisera may be preferred to increase the likelihood of reaction against all antigenic variants. AgELISAs have been developed for detection of *Anaplasma* (Trueblood *et al.,* 1991), BVDV (Mignon *et al.,* 1991), rinderpest and PPR (Libeau *et al.,* 1994). Related antigen-capture methods using immunomagnetic beads are now important and well accepted methods for detecting certain bacterial infections, including *Listeria, Salmonella* and *Escherichia coli.* The principle of this technology relies on immunomagnetic separation, i.e. using small super-paramagnetic particles or beads coated with antibodies against surface antigens of cells. Both intact bacteria and their soluble antigenic determinants can be detected after magnetic extraction from the test sample using a second antibody in a sandwich format. As solid surface-free binding, the antigen-capture assays using immunomagnetic beads can enhance the kinetics of an antigen-antibody reaction. As a result, both nonspecific binding and the incubation time are reduced.

2. Quantitative immunoassays (radioimmunoassay and quantitative enzyme immunoassay)

Quantitative ELISA (qELISA) is not used specifically for disease detection. However, it is widely used for measurement of the concentration of specific proteins (thyroxin, steroids) or other proteins or haptens in the body or body fluids. Hence, these methods are often used for diagnosis of hormonal disorders, mainly in animal reproduction. The principle is very similar to the one of the cELISA. The plates are coated with antibodies against the substance that is to be measured. The competitive substance is labelled with a marker (an isotope in a radioimmunoassay and an enzyme or biotin in an enzyme immunoassay) and is titrated to a concentration that will saturate the coating antibodies. A set of standards of known concentration is added to the wells of the ELISA plate. As the concentration of the standard increases, the labelled competitor is increasingly displaced in appropriate wells, resulting in reduced colouration at the end of the reaction. Based on this correlation, a standard curve is drawn, showing the discoloration (percentage of displacement) as a function of the known concentration.

Once the standard curve is drawn, the percentage of displacement caused by the unknown sample is measured and the concentration of the targeted substance read via the standard curve.

3. Immunohistochemistry

As an adjunct to the isolation of causative organisms from tissue, immunohistochemistry has become a standard tool for identification of pathogens, and also for confirmation of the results obtained using high-tech diagnostic technologies (e.g. mass tag PCR). The *in situ* detection of antigens in fixed tissues offers a number of advantages over other diagnostic techniques. These advantages are: (i) convenience of sample submission; (ii) safe handling of potential human pathogens; (iii) retrospective studies of stored specimens; (iv) rapidity; and (v) the detection of nonviable organisms (Haines & Clark, 1991). Immunohistochemistry is also used for the detection of abnormal prion protein (PrP^{Sc}) in brain tissue to confirm scrapie, BSE and other transmissible spongiform encephalopathies, and has proved to be more sensitive than standard histopathological examination (Thorgeirsdottir *et al.,* 2002). As the number of MAbs to defined antigens increases, the use of immunohistochemistry for the identification of organisms and other specific markers for autoimmune diseases and neoplasia is increasing. The limiting step in the application of immunohistochemistry is identifying an appropriate MAb/antigen combination that will bind in formalin-fixed tissues. This may be overcome by using frozen sections or employing antigen retrieval techniques (e.g. proteolytic enzyme digestion, microwaving) before immunostaining.

4. Immunochromatography

Immunochromatograhy provides a convenient method for detection of antigens in several minutes without special apparatus (Lyoo *et al.,* 2005). The sample is applied on one end of a filter in which antibody is immobilised and microbeads (such as colloidal gold) conjugated with antibody are applied. The antigen in the sample forms an immunocomplex with the antibody labelled with colloidal gold. This complex moves along with the liquid sample, and makes a contact with the antibody immobilised on the membrane, where it forms an immuno-complex with the immobilised antibody, generating a colored product that can be visualised by eye.

5. Proteomics

The proteome is the total complement of proteins expressed within a cell, a tissue or an organism and proteomics is the study of proteins, including their expression level, post-translational modification and interaction with other proteins, on a large scale. As not all proteins are expressed at all times, but are dependent on physiological and environmental factors, proteomics can provide an excellent overall view of disease processes at the protein level. Because the application of proteomics to novel drug discovery promises huge economic returns, companies all over the world have rapidly poured resources into this new research field (Brower, 2001).

Many methods used in proteomics, including two-dimensional gel electrophoresis (2DGE) and mass spectrometry (MS) were established many years ago. However recent advances in MS techniques, together with whole genome sequencing and the development of powerful bioinformatics and robotics platforms, have revolutionised protein identification. The general principle of proteomics is that proteins are separated, usually by 2DGE on polyacrylamide gels, then protein spots are excised, digested with trypsin, and the resultant peptides analysed by MS. The masses of these peptides are then compared with the predicted masses of peptides derived by computational analyses of genome databases, resulting in gene identification. MS can also be used to deduce the amino acid sequence of peptides and to characterise post-translational modifications such as glycosylation or phosphorylation. 2DGE has some drawbacks, particularly for the separation of hydrophobic proteins, and other separation techniques based on liquid chromatography are now finding favour for certain applications. Nevertheless, 2DGE is the method of choice for creating quantitative maps of protein expression and many thousands of proteins can be analysed in a short space of time.

Alterations in the proteome of body tissues or of fluids such as serum, urine or cerebro-spinal fluid can be measured directly so changes that occur in a disease state can be accurately pinpointed. As well as identifying molecules that may be targets for novel therapies, this approach is a very powerful tool for early-stage diagnosis of disease. The best-established clinical applications of proteomics so far are in the identification of markers for the early diagnosis of cancers, such as bladder cancers, in urine (Rasmussen *et al.,* 1996). However, considerable research efforts are also ongoing in other areas such as heart disease, Alzheimer's disease and insulin-dependent diabetes.

The use of proteomics for the diagnosis of infectious disease is in its infancy but may prove to be of considerable importance. For example, definitive diagnosis of chronic hepatitis B virus (HBV) infection still relies on liver biopsy, but proteomic analysis of serum samples shows that the expression of at least seven serum proteins is changed significantly in chronic HBV patients. Similarly, the ante-mortem differential diagnosis of Creutzfeldt-Jakob disease (CJD) may be aided by proteomics as preliminary data show that seven proteins in cerebro-spinal fluid (CSF) are differentially expressed between patients with variant or sporadic CJD (Choe *et al.,* 2002).

An extremely useful application of proteomics to the diagnosis of infectious disease is in the identification of novel diagnostic antigens by screening serum from infected and uninfected individuals against immunoblotted, 2DGE mapped proteomes of infectious agents. Using this type of approach with human sera, nine new potential immunodiagnostic antigens have been identified in *Helicobacter pylori* (Goebel *et al.,* 2002).

Within the veterinary field, proteomics-based research projects are now underway and these will undoubtedly yield novel diagnostic tools for the future. Proteome maps are being derived for a range of veterinary pathogens including bacteria (Hughes *et al.,* 2002; Mujer *et al.,* 2002), protozoa (Rout & Field, 2001) and nematodes (Yatsuda *et al.,* 2003).

B3. DETECTION OF ANTIBODIES

1. Complement fixation test

Complement is a thermolabile system of over 25 proteins present in the blood that are sequentially activated by antigen/antibody complexes to assist in eliminating pathogens. The complement fixation test (CFT) relies on the property of some antibodies to use complement as a mediator. The test is based on two independent reactions, each requiring complement as a mediator: (i) the 'main' reaction in which the test serum (heat-inactivated to destroy the complement) is incubated with an external source (usually rabbit or guinea pig) of complement and the antigen; and (ii) the 'helper' (indicator) reaction, where anti-erythrocyte antibodies ('haemolysin') are incubated with washed sheep erythrocytes in predefined concentration. If the tested serum in the main reaction is positive (specific for the targeted antigen), the complement will have been used and the whole mixture will therefore lack active complement. If the reaction is negative, the complement will not have been spent and the mixture will contain active complement. After an appropriate incubation period, a defined volume from the 'main' reaction mixture is added to the 'helper' reaction mixture. In the case of positive reaction (no active complement in the 'main' mixture), the erythrocytes in the 'helper' reaction will not be haemolysed and will form a discrete round button on the bottom of the tube (or well in tests where a microplate format is used). The supernatant in these tubes (wells) will be clear. In the case of a negative reaction (complement in the 'main' mixture is active); the erythrocyte in the 'helper' reaction will be haemolysed. In this case there will be no formation of the discrete button on the bottom of the tube (or well in tests where microplate format is used) will occur. The supernatant in these tubes (wells) will be redish and turbid. The CFT can be used for qualitative and semiquantitative detection of specific antibodies. It is still widely used for detection of antibodies against brucellosis in cattle, sheep and goats (European Commission [2001]), Q fever, contagious bovine pleuropneumonia (CBPP) and several other diseases.

2. Indirect ELISA (iELISA)

In an iELISA, the target antigen (whole or purified) is bound to the solid phase in an ELISA plate. Serum samples at an appropriate dilution are added. If specific antibodies against the coated antigen are present, they will bind to the antigen. The ELISA plates are washed to remove any unbound antibodies. Anti-imunoglobulin antisera conjugated with an enzyme (usually a peroxidase), specific for the immunoglobulin of the animal species examined are then added. If specific antibodies against the coated antigen have been present in the first phase, the conjugated anti-immunoglobulins will bind to them and will not be removed during the second washing step. The substrate buffer is then added. In positive cases (presence of specific antibodies) the colour of the substrate buffer will change. The colour development is measured at a defined wavelength using a spectrophotometer and is proportional to the level of antibodies present in the sample.

3. Competitive ELISA (cELISA)

The cELISA is an immunoassay that can be used to detect or quantify antibody or antigen using a competitive method. The cELISA for detection of specific antibodies has largely replaced the iELISA for large-scale screening and sero-surveillance. The cELISA offers significant advantages over an iELISA, as samples from many species may be tested without the need for species-specific enzyme-labelled conjugates for each species under test. Many antigens are extremely difficult or time consuming to purify. When used in an indirect assay, they can produce high background values because of nonspecific binding. However, relatively crude antigens may be used in the cELISA provided the 'detecting antibody' has the desired specificity. The principle of a competitive assay for the detection of antibodies is competition between the test serum and the detecting antibody. Specific binding of the detecting antibody is detected using an appropriate anti-species conjugate. A reduction in the expected colour obtained is caused by binding of antibodies present in the test serum with the antigen, which therefore prevents binding of the specific detecting antibody.

4. Blocking ELISA (bELISA)

The basic setup in the bELISA is similar to that described for the AgELISA (paragraph B2.1.). Antibodies specific to the targeted antigen are bound to the solid phase of the ELISA plate. The antigen, prior to adding to the antibody coated plate, is incubated with the samples. If the samples contain antibodies against that antigen, they will bind (block) to the antigen during this incubation. When added to the wells, the blocked antigen will be unable to bind to the coating antibodies. Consequently, when the detecting antibody is added to the wells it will not be able to recognise any bound antigen (no colour development). In contrast, if the sample is derived from a negative case, binding will occur and there will be colour development.

5. Production of antigens by recombinant DNA technology

Advances in molecular biology and genetics in the 1970s initiated the development of recombinant DNA technology. Since then the impact of this technology is such that it plays a vital role in scientific research as well as in the diagnosis and treatment of disease. Recombinant DNA technology simply refers to the transfer of genes from one organism into another: literally the recombination of DNA from different sources. The objectives of recombinant DNA technology include identifying genes, isolating genes, modifying genes, and re-expressing genes in other hosts or organisms. These steps permit scientists and clinicians to identify new genes and the proteins they encode them, to correct endogenous genetic defects, and to manufacture large quantities of specific gene products such as hormones, antigens for use in vaccines, and other proteins produced by biological agents of interest. Of particular value is the degree of specificity attainable in diagnostic tests by the use of recombinant protein.

Native proteins are perhaps the ideal antigens, providing sequence-specific and surface structural epitopes. Many current diagnostic tests require test antigens that need to be continuously produced from cell culture or harvested from an infected animal. Such antigen preparations are expensive and often have a short shelf-life, with each new batch of antigen requiring standardisation. Natural proteins are rarely available in a completely pure form, and antibodies often develop against contaminating polypeptides that can lead to false-positive results. Recombinant DNA technology produces antigens that offer many advantages over antigens isolated from other biological sources. These advantages include a high purity, high specific activity and as the protein is synthesised in genetically modified laboratory-grown cells, each preparation of the protein product is identical to the previous preparation, ensuring batch-to-batch consistency. When recombinant antigens are used in combination with the cELISA format, purification of the recombinant antigen from the lysate may not be necessary as the specificity of the cELISA resides mainly in the MAb used.

An outline of the procedure for the production of an antigen by recombinant DNA technology is as follows. The identification of an antigen of potential diagnostic or scientific significance is achieved through the study of the antibody response of the host to the proteins of the organism in question. Immunodominant antigens, defined proteins of the organism against which the host responds with the highest potential antibody titre, are of particular interest as they are likely to be major stimuli of cellular and humoral immunity against the disease of interest. Antigen recognition studies are widely used to identify biologically relevant, immunodominant antigens for use in generating MAbs as well as in vaccine development. Once a protein of interest has been identified, the gene encoding the protein is generated using messenger RNA (mRNA) from the organism as a template for making cDNA. This method of cloning the gene encoding the protein of interest requires a prior knowledge about the gene sequence, either directly from the organism of interest or through the use of gene sequences from closely related species. An alternative method, when gene sequence data are not available, is the generation of recombinant libraries from the genomic DNA of the organism or from cDNA synthesised from mRNA. Fragments of the recombinant libraries can be cloned into an expression system, which may be prokaryotic or eukaryotic, and the gene library screened for expression of the protein.

There is a wide choice of expression systems. Protein may be expressed in bacteria, usually *E. coli* (Rasmussen *et al.,* 1996), yeast (Carter *et al.,* 1987), insect cells using baculovirus (Stewart & Possee, 1993), or in eukaryotic cells by infection with appropriate viral vectors (Smith *et al.,* 1983) or by permanent transfection. Differences in glycosylation when prepared in bacterial, insect or mammalian cell cultures can modify protein structure and its reactivity with antibody. Antigen may need to be extracted from the cell or it may be secreted. Purification is often, but not always, necessary. An upcoming trend in the production of antigens for use in assays is in the development of synthetic peptide antigens. This allows antigens to be tested as diagnostic reagents based on the gene sequence, expression of the whole protein being unnecessary, thus shortening the process.

Genome sequences of hundreds of bacteria and thousands of viruses have already been determined. An antigen gene can easily be cloned with PCR technology, using primers designed from the nucleotide sequence of closely related species (Rappuoli & Covacci, 2003). The gene can also be expressed and its product can be purified using tag peptide. The antigenicity of the gene products can then be determined. Systematic screening of the antigen gene in silico, from genome sequence data, accelerates the development of diagnostic kits and vaccine (Tortorella *et al.,* 2000).

6. Developing technologies for protein detection

The following protein-detection technologies are in the developmental stage:

i) Multiplex ELISA platforms that can be deployed to create “disease package assays” (BVDV, Infectious bovine rhinotracheitis [IBR], parainfluenza virus 3 [PI3] and respiratory syncytial virus [RSV]). The test uses the same principle as the conventional ELISA, adapted into a micro-format.

ii) Time-resolved fluorescence resonance energy transfer (TR-FRET) used as a source of signal generation. The labelling of the conjugate and the antigen is performed using two fluorophore dyes. The principle has been tested for competitive and sandwich format.

iii) “Potentiometric ELISA” based on production of an electrical, instead of a colour signal, requiring minimal sample preparation and producing results within 2 to 15 minutes.

iv) A SERS assay (using colloidal gold as a ligand) for detection of DNA/RNA and proteins in immunological reactions using piezoelectric effect, as well as amplification of the signal using magnetic capture.

v) Multiplex protein assay based on signal generation using piezoelectric effect and the oscillation modulation caused by specific analyte binding. The assay has been developed for rapid result generation (60 seconds), multiplex capacity, qualitative and quantitative measurements, does not need reagents and/or incubations. The device is the size of a mobile phone, and integrated wireless transmission of the results can be used to expedite data analysis. The principle of the work has been scientifically proven and preliminary investigations on its applicability to avian influenza diagnosis are in progress.

B4. IMPLICATIONS OF THE NEW TECHNOLOGIES

There are a number of trends in diagnostic technologies that will have an impact on the way in which disease diagnosis will be approached in the future, affecting the laboratory environment, data analysis and disease control:

i) The global development of chip technologies has led to a strong trend towards miniaturisation of the test format in both molecular and protein detection assays. The test formats range from several millimetres to several centimetres.

ii) Although miniaturised, multiplex platforms can be developed that are capable of detecting from several up to thousands of pathogens in a single sample.

iii) Miniaturisation of the test format is being accompanied by similar miniaturisation of laboratory equipment, enabling on-site testing. This trend is resulting in the use of sophisticated equipment, previously only used in the laboratory, in the field, facilitating the rapid and early diagnosis of infectious diseases, as well as the modes of reaction of appropriate Competent Authorities.

iv) The development of alternative sources of signal generation/amplification, replacing light with mass measurement, piezoelectric effect or concentration of the ligand will lead to the development of a whole new platform of technologies.

vi) Although development of new technologies can often mean improved capability, consideration should always be paid to the actual value and the role of the confirmatory test in diagnosis!

viii) As the diagnostic platforms are continuously changing in the ways described above, several components of the disease control chain will be affected. Appropriate communication technologies/information systems will need to be developed in order to systematically collect, store and analyse large datasets produced by the new technologies in a relatively short time. There is likely to be an increasing trend towards real-time inputs of results via mobile telephones or the Internet that will require relevant development of IT and data-handling systems.

ix) Appropriate infrastructure and preparedness have to be an integral component of technology update. This will include distance training in testing in the field (sample collection and preparation, testing, interpretation of the test results, sample processing for dispatch to reference laboratories and reporting to appropriate authorities).

x) Adaptation of national contingency plans towards simultaneous management of multiple diseases, as well as management of new, unexpected diseases.

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ADVICE FOR MEMBER COMMENTS

Chapter Number and Title: 2.1.1. Anthrax

Country making the comments

Date

It would be appreciated if the following guidance notes are followed in making a reply:

1. *Comments may be general or specific, but specific comments are usually more valuable. General comments should be such that some conclusion and action can be taken in response to them. For example, it is of little help to make statements such as "This test is no longer used in our laboratory", without indicating the reasons and what test is used instead.*

2. *Specific comments should be identified, by indicating the line number in the text, to facilitate the making of editorial changes.*

3. *The pointing out of typing or technical errors is welcome, but the correct word or figure should be indicated. For example, it is of little help to say that 0.8 M is too high, if the preferred value is not indicated.*

4. *Bear in mind that the introductory chapters are not intended to be exhaustive, and indeed none of the chapters can give a completely comprehensive cover of the subject, otherwise the* Terrestrial Manual *would be too long. However, assistance in indicating priorities is always helpful.*

5. *The* Terrestrial Manual *is intended for world-wide use. The chapters need to reflect the development of new technology, but the old established methods, usually requiring less sophisticated apparatus, have to be retained. New technology should not be described in detail until it has gained wide acceptance as a reliable method.*

General Comments

Specific Comments (add continuation sheets if required)
line:

PART 2

OIE LISTED DISEASES AND OTHER DISEASES OF IMPORTANCE TO INTERNATIONAL TRADE

SECTION 2.1.

MULTIPLE SPECIES

CHAPTER 2.1.1.

ANTHRAX

SUMMARY

Definition of the disease*: Anthrax is primarily a disease of herbivorous animals, although all mammals, including humans, and some avian species can contract it. Mortality can be very high, especially in herbivores. The aetiological agent is the spore-forming, Gram-positive rod-shaped* Bacillus anthracis*. The disease has world-wide distribution and is a zoonosis.*

Description of the disease: *The disease is mediated by exotoxins. Peracute, acute, subacute and, rarely, chronic forms of the disease are reported. Ante-mortem clinical signs may be virtually absent in peracute and acute forms of the disease. Subacute disease may be accompanied by progressive fever, depression, inappetence, weakness, prostration and death. Acute, subacute, and chronic disease may show localised swelling and fever. ~~and~~ In chronic disease, the only sign may be enlarged lymph glands.*

Identification of the agent: Bacillus anthracis *is readily isolated in relatively high numbers from blood or tissues of a recently dead animal that died of anthrax, and colony morphology of* B. anthracis *is quite characteristic after overnight incubation on blood agar. The colony is relatively large, measuring approximately 0.3–0.5 cm in diameter. It is grey-white to grey, non-haemolytic with a rough, ground-glass appearance and has a very tacky, butyrous consistency. The vegetative cells of* B. anthracis *are large, measuring 3–5 µm in length and approximately 1 µm in width. Ellipsoidal central spores, which do not swell the sporangium, are formed at the end of the exponential cell growth phase. The cells stain strongly Gram positive, and long chains are often seen* in vitro*, while paired or short chains are seen* in vivo*. Visualisation of the encapsulated bacilli, usually in large numbers, in a blood smear stained with polychrome methylene blue* (*M'Fadyean reaction*) *is fully diagnostic.*

Serological tests: *Antibody detection in serum from infected animals is rarely used for diagnostic purposes and is essentially a research tool. The predominant procedure ~~today~~ used is the enzyme-linked immunosorbent assay* (*ELISA*)*.*

Requirements for vaccines ~~and diagnostic biologicals~~: *The most widely used livestock anthrax vaccine developed by Max Sterne in 1937, is a live, non-encapsulated, spore former held in suspension. In Russia and Eastern Bloc countries, an equivalent type of vaccine is used* (*strain 55*)*. ~~The Pasteur vaccine is no longer used in Italy.~~ A new vaccine, Carbosap, has been developed that retains both plasmids and exhibits very low virulence. A list of producers is given in the World Health Organization anthrax guidelines.*

A. INTRODUCTION

Anthrax, an acute bacterial disease of primarily herbivores, is transmissible to humans. The aetiological agent, *Bacillus anthracis*, is a Gram-positive spore-forming rod-shaped bacterium. Anthrax is known by many names around the world including charbon, woolsorters disease, ragpickers disease, malignant carbuncle, and malignant pustule.

Animals become infected by ingesting spores or possibly by being bitten by flies that have fed on an infected animal or carcass. Infected animals are usually found dead as death can occur within 24 hours. A careful post-mortem examination of recently dead animals may show any number of lesions, none of which is pathognomonic or entirely consistent. To avoid environmental contamination, post-mortem examinations of carcasses of animals suspected to have died of anthrax ~~is~~ are discouraged. Lesions most commonly seen are those of a generalised septicaemia often accompanied by an enlarged spleen having a 'blackberry jam' consistency and poorly clotted blood. Haemorrhage from the nose, mouth, vagina and/or anus at death is not a common sign.

Gram-positive rod-shaped *Bacillus anthracis* is the only obligate pathogen within the genus *Bacillus.* Most of the other species of *Bacillus* are common ubiquitous environmental saprophytes, although a number, notably *B. cereus, B. licheniformis* and *B. subtilis*, are occasionally associated with food poisoning in humans and with other clinical manifestations in both humans and animals.

More than 95% of human anthrax cases take the cutaneous form and result from handling infected carcasses or hides, hair, meat or bones from such carcasses. *Bacillus anthracis* is not invasive and requires a lesion to infect. Protection for veterinarians and other animal handlers involves wearing gloves, and other protective clothing when handling specimens from suspected anthrax carcasses and never rubbing the face or eyes. The risk of gastrointestinal anthrax may arise if individuals eat meat from animals infected with anthrax.

The risk of inhaling infectious doses becomes significant in occupations involving the processing of animal by-products for manufacturing goods (industrial anthrax). These include the tanning, woollen, carpet, bone processing, and other such industries, where the potential for aerosolisation of substantial numbers of spores increases the risk of exposure to infectious doses. It is important that industrial workers use appropriate personal protective gear and follow standard operating procedures.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Demonstration of encapsulated *B. anthracis* in smears of blood or tissues from fresh anthrax-infected carcasses and growth of the organism on blood agar plates is relatively uncomplicated and within the capability of most bacteriology laboratories. Difficulty may be encountered in the case of pigs and carnivores in which the terminal bacteraemia is frequently not marked, or in animals that received antibiotics before death.

Recovery of *B. anthracis* from old decomposed carcasses, processed specimens (bone meal, hides), or environmental samples (contaminated soil) is ~~also~~ often difficult, requiring demanding and labour-intensive procedures.

a) Culture and identification of *Bacillus anthracis*

i) Fresh specimens

Bacillus anthracis grows readily on most types of nutrient agar, however, 5–7% horse or sheep blood agar is the diagnostic medium of choice. Blood is the primary clinical material to examine. Swabs of blood, other body fluids or swabs taken from incisions in tissues or organs can be spread over blood agar plates. After overnight incubation at 37°C, *B. anthracis* colonies are grey-white to grey, 0.3–0.5 mm in diameter, non-haemolytic, with a ground-glass moist surface, and very tacky when teased with an inoculating loop. Tailing and prominent wisps of growth trailing back toward the parent colony, all in the same direction, are sometimes seen. This characteristic has been described as a 'medusa head' appearance. Confirmation of *B. anthracis* should be accomplished by the demonstration of a capsulated, spore-forming, Gram-positive rod in blood culture. Absence of motility is an additional test that can be done.

Anthrax-specific phages were first isolated in the 1950s, and the specifically named gamma phage was first reported in 1955 (Brown & Cherry, 1955) and quickly became the standard diagnostic phage for anthrax. Gamma phage belongs to a family of closely related anthrax phages (World Health Organization [WHO], 2008).

~~The susceptibility of *B. anthracis* to the gamma bacteriophage was first described by Brown & Cherry in 1955 (3). The phage is available from various national central veterinary laboratories and anthrax reference laboratories.~~

Two tests for confirming the identity of *B. anthracis* are gamma phage lysis and penicillin susceptibility. The typical procedure for these tests is to plate ~~simply to streak~~ a lawn of suspect *B. anthracis* ~~with the suspect organism~~ on a blood or nutrient agar plate~~, or portion of a plate (several tests can be done on one plate),~~ and place a 10–15 µl drop of the phage suspension on one side of the lawn ~~streaked area~~ and ~~place~~ a 10-unit penicillin disk to the other side. Allow the drop of phage suspension to soak into the agar before incubating ~~and incubate~~ the plate at 37°C. A control culture, ~~should be included;~~ e.g. the Sterne vaccine or

the NCTC strain 10340, ~~can be used for this~~ should be tested at the same time as the suspect culture to demonstrate the expected reaction for gamma phage lysis and penicillin susceptibility. If the suspect culture is *B. anthracis*, the area under the phage will be devoid of bacterial growth, because of lysis, and a clear zone will be seen around the penicillin disk ~~after overnight incubation~~ indicating antibiotic susceptibility. ~~It should be~~ Noted that ~~phage-resistant *B. anthracis* isolates are encountered occasionally; similarly, there are a few reports in the literature of penicillin-resistance~~ some isolates of *B. anthracis* may be phage resistant or penicillin resistant. As the performance of the gamma phage lysis assay may be affected by the density of bacterial inoculum, Abshire *et al.* (2005) recommend streaking the suspect culture on the agar plate over several quadrants instead of using a lawn format and inoculating a drop of gamma phage on the first and second quadrants on the plate.

Phage suspensions may be obtained from central veterinary laboratories or central public health laboratories.

The phage can be propagated and concentrated by the following protocol. Store phage at 2–4°C and do not freeze phage as it will quickly become non-viable.

Stage one

i) Spread a blood agar (BA) plate of the Sterne vaccine strain of *B. anthracis*. Incubate overnight at 37°C.

ii) Inoculate approximately 10 ml of nutrient broth (NB) with growth from the BA plate and incubate at 37°C for approximately 4 hours or until just cloudy, then refrigerate.

iii) Spread 100 µl of the culture from step ii on three pre-dried BA plates and incubate at 37°C for 30–60 minutes.

iv) Spread 100 µl of the phage suspension to be amplified over the same plates. Incubate at 37°C overnight.

v) Harvest the phage-lysed growth on the BA plate in 5 ml of NB followed by a second 'wash' of 5 ml NB. Incubate at 37°C overnight.

vi) Filter (0.45 µm) and count by dropping 20 µl drops (three drops per dilution) of tenfold dilutions of the filtrate in saline onto lawns of the *B. anthracis* culture prepared as in step iii.

Stage two

This is essentially the same procedure as Stage one, only uses the filtrate from step vi to harvest the phage from the plates.

vii) Prepare three Sterne strain lawns on BA, as in step iii. Incubate at 37°C for 30–60 minutes.

viii) Spread 100 µl phage from step vi. Incubate at 37°C overnight.

ix) To 9 ml of filtrate from step vi, add 1 ml of 10× concentrated NB.

x) Harvest the phage from step viii with 5 ml of the solution from step ix, followed by a second 5 ml wash with the rest of the solution from step ix.

xi) Add 10 ml of 1× NB.

xii) Incubate at 37°C overnight, filter and count.

Stage three

xiii) Inoculate 100 ml of brain–heart infusion broth with approximately 2.5 ml of the culture from step ii. Incubate on a rotary shaker at 37°C until just turbid.

xiv) Add the 20 ml of filtrate from step xii and continue incubation overnight.

xv) The resultant filtrate is checked for sterility and titrated in ten-fold dilutions on lawns of the vaccine strain as in step vi to determine the concentration of the phage. This should be of the order of 10^8–10^9 plaque forming units per ml.

ii) Capsule visualisation

Virulent encapsulated *B. anthracis* is present in tissues and blood and other body fluids from animals that have died from anthrax. The bacteria should be looked for in smears of these specimens that have been dried, fixed either using heat or by dipping the smear in 95–100% alcohol for about 1 minute and air dried and then stained with polychrome methylene blue (M'Fadyean's reaction). The capsule stains pink, whereas the bacillus cells stain dark blue. The cells are found in pairs or short chains and are often square-ended (the chains are sometimes likened to a set of railway carriages – so-called 'box-car' appearance). Gram and Giemsa stains do not reveal the capsule. The capsule is not present on *B. anthracis* grown aerobically on nutrient agar or in nutrient broths, but can be seen when the virulent bacterium is cultured for a few hours in

a few millilitres of blood (defribrinated horse or sheep blood seems to work best). Alternatively, the capsule is produced when the virulent *B. anthracis* is cultured on nutrient agar containing 0.7% sodium bicarbonate and incubated in the presence of CO_2 (20% is optimal, but a candle jar works well). The agar is prepared by reconstituting enough nutrient agar base powder for 100 ml of agar in 90 ml of water. Autoclave and cool to 50°C in a water bath. Add 10 ml of a filter-sterilised (0.22–0.45 µm filter) 7% solution of sodium bicarbonate. Mix and pour into Petri dishes. The encapsulated *B. anthracis* will form mucoid colonies and the capsule can be visualised by making thin smears on microscope slides, fixing, and staining with polychrome methylene blue (M'Fadyean's stain).

Polychrome methylene blue ~~is~~ can be prepared as follows: 0.3 g of methylene blue is dissolved in 30 ml of 95% ethanol; 100 ml of 0.01% potassium hydroxide (KOH) is mixed with the methylene blue solution. Ideally, this should be allowed to stand exposed to the air, with occasional shaking, for at least 1 year to oxidise and mature. Addition of K_2CO_3 (to a final concentration of 1%) hastens the 'ripening' of the stain, but before it is regarded as diagnostically reliable, its efficacy should be established by testing it in parallel with an earlier, functional batch of stain on *bona fide* samples. It has been found that stains that give positive reactions with cultures of *B. anthracis* cultured artificially in horse blood sometimes do not give positive results in the field.

In making smears for staining, only small drops of blood or tissue fluid are needed and a thin, small smear is best. After fixing (either using heat or by dipping the smear in 95–100% alcohol for about 1 minute) and drying, a small (approximately 20 µl) drop of stain is placed on the smear and spread over it with an inoculating loop. After 1 minute, the stain is washed with water, blotted, air-dried and observed initially using the ×10 objective lens under which the short chains appear like short hairs; once found, these can be observed under oil immersion (×1000) for the presence of the pink capsule surrounding the blue/black-staining bacilli. To avoid laboratory contamination, the slide and blotting paper should be autoclaved or left for some hours in a 10% sodium hypochlorite solution.

iii) Other specimens

Identification of *B. anthracis* from old, decomposed specimens, processed materials, and environmental samples, including soil, is possible but these samples often have saprophytic contaminants that outgrow and obscure *B. anthracis* on non-selective agars. The following procedure is suggested:

a) The sample is blended in two volumes of sterile distilled or deionised water and placed in a water bath at 62.5 ± 0.5°C for 30–60 minutes. Turnbull *et al.* (2007) have demonstrated that heat activation of spores can be conducted at a temperature range of 60–70°C with holding times not exceeding 15–30 minutes for best recovery.

b) Tenfold dilutions to 10^{-2} or 10^{-3} are then prepared. From each dilution, 10–100 µl are plated on to blood agar and optionally 250–300 µl on to PLET agar (polymyxin, lysozyme, EDTA [ethylene diamine tetra-acetic acid], thallous acetate) (Knisely, 1966; WHO, 2008). All plates are incubated at 37°C.

c) Blood agar plates are examined for typical colonies as previously described after overnight incubation, and the PLET plates are examined after 40–48 hours. Confirmation of the identity of suspect colonies as *B. anthracis* is done as described above.

PLET medium (Knisely, 1966; WHO, 2008) is prepared by using heart-infusion agar base (DIFCO) made up to the manufacturer's instructions with the addition of 0.25–0.3 g/litre EDTA and 0.04 g/litre thallous acetate. ~~(*NOTE: thallous acetate is poisonous and should be handled with care.*)~~ The mixture is autoclaved and uniformly cooled to 50°C before adding the polymyxin at 30,000 units/litre and lysozyme at 300,000 units/litre. After mixing thoroughly, the agar is dispensed into Petri dishes.

Reports of procedures for direct detection of *B. anthracis* in soils and other environmental specimens using the polymerase chain reaction (PCR) are emerging. None of these has become routinely applicable at the present time.

Animal inoculation may be considered for recovery of *B. anthracis* if all other methods fail. Examples of when this might occur are specimens from animals that received antibiotic therapy before death or environmental samples containing sporostatic chemicals. Due to the increasing concern to eliminate the use of animals for biological testing, this approach should be used as a last resort and only if justified. Adult mice or guinea-pigs are the animals of choice. If the samples involved are soils, the animals should be pretreated, the day before testing, with both tetanus and gas gangrene antiserum. The samples are prepared as described for culturing, including heat-shocking at 62.5°C for 15 minutes. Mice are injected subcutaneously with 0.05–0.1 ml; guinea pigs are inoculated intramuscularly with up to 0.4 ml (0.2 ml in each thigh muscle). Any *B. anthracis* present will result in death in 48–72 hours and the organism can be cultured from the blood as described above.

b) Immunological detection and diagnosis

It needs to be borne in mind that *B. anthracis* is antigenically very closely related to *B. cereus,* which is considered a ubiquitous component of the environmental microflora. The only unshared antigens that lend themselves to differentiating these two species by immunological approaches are the anthrax toxin antigens, produced during the exponential phase of growth, and the capsule of *B. anthracis*. This places considerable constraints on the extent to which immunological methods can be used in routine detection methodology.

i) Ascoli test

Ascoli (1911) published a procedure for the detection of thermostable anthrax antigen in animal tissue being used for by-products. This uses antiserum raised in rabbits to produce a precipitin reaction. The test lacks high specificity, in that the thermostable antigens of *B. anthracis* are shared by other *Bacillus* spp., and is dependent on the probability that only *B. anthracis* would proliferate throughout the animal and deposit sufficient antigen to give a positive reaction. ~~Nowadays, it~~ This test appears to be used only in Eastern Europe ~~only~~.

To perform the Ascoli test, put approximately 2 g of sample in 5 ml of saline containing 1/100 final concentration of acetic acid and boil for 5 minutes. The resultant solution is cooled and filtered through filter paper. A few drops of rabbit antiserum (see preparation below) are placed in a small test tube. The filtrate from the previous step is gently layered over the top of the antiserum. A positive test is the formation of a visible precipitin band in under 15 minutes. Positive and negative control specimen suspensions should be included.

Antiserum is prepared in rabbits by the subcutaneous inoculation of Sterne anthrax vaccine on days 1 and 14. On days 28 and 35, the rabbits receive 0.5 ml of a mixture of several strains of virulent *B. anthracis* not exceeding 10^5 colony-forming units (CFU)/ml suspended in saline. Alternatively, the live virulent bacteria can be inactivated by prolonged suspension in 0.2% formalised saline, but the antigen mass needs to be increased to 10^8–10^9 CFU/ml. The suspension should be checked for inactivation of the *B. anthracis* before animal inoculation by culture of 0.1 ml into 100 ml of nutrient broth containing 0.1% histidine and, after incubation at 37°C for 7 days, subculture on to blood or nutrient agar. The dose regimen for the formalised suspension after initial vaccination on days 1 and 14 is increasing doses of 0.1, 0.5, 1, and 2 ml given intravenously at intervals of 4–5 days. Following either procedure, a test bleed at 10 days after the last injection should determine whether additional 2 ml doses should be administered to boost the precipitin titre.

ii) Immunofluorescence

While some success has been achieved with immunofluorescence for capsule observation in the research situation (Ezzel & Abshire, 1996), it does not lend itself to routine diagnosis.

c) Confirmation of virulence with the polymerase chain reaction

~~Full~~ Confirmation of virulence can be carried out using the PCR. The following instructions are taken from the WHO (2008). Template DNA for PCR can be prepared from a fresh colony of *B. anthracis* on nutrient agar by suspension of a loop of growth in 25 µl sterile deionised (or distilled) water and heating to 95°C for 20 minutes. Following cooling to approximately 4°C, and brief centrifugation, the supernatant can be used for the PCR reaction.

Examples of suitable primers (Beyer *et al.,* 1996; Hutson *et al.,* 1993) for confirming the presence of the pXO1 and pXO2 plasmids are given in the table below.

Target	Primer ID	Sequence 5'–3'	Product size	Concentration
Protective antigen (PA)	PA 5 3048–3029	TCC-TAA-CAC-TAA-CGA-AGT-CG	596 bp	1 mM
	PA 8 2452–2471	GAG-GTA-GAA-GGA-TAT-ACG-GT		
Capsule	1234 1411–1430	CTG-AGC-CAT-TAA-TCG-ATA-TG	846 bp	0.2 mM
	1301 2257–2238	TCC-CAC-TTA-CGT-AAT-CTG-AG		

PCR can be carried out in 50 µl volumes using the above primers, 200 µM each of dATP, dCTP, dTTP and dGTP, 1.5 mM $MgCl_2$ and 2.5 units of ~~ampli Taq~~ AmpliTaq® DNA polymerase[1], all in NH_4 buffer, followed

1 Applied Biosystems http://www.appliedbiosystems.com.~~(https://products.applied biosystems.com/ab/en/US/adirect/ab?cmd=catNavigate2&catID=601622~~

by the addition of 5 µl of template DNA. A 2% agarose gel has been found to work best with these small fragments.

Alternatively, 'Ready-To-Go™' beads are available ~~from GE Healthcare Pharmacia Biotech~~[2]. These are premixed, predispensed, dried beads, stable at room temperature, containing all the necessary reagents, except primer and template, for performing 25 µl PCR reactions. The template can be added in a 2.5 µl volume.

The following PCR cycle can be used: 1 × 95°C for 5 minutes; 30 × 95°C for 0.5 minute followed by 55°C for 0.5 minute followed by 72°C for 0.5 minute; 1 × 72°C for 5 minutes; cool to 4°C.

It should be noted that~~, in use for some years now in an anthrax reference facility,~~ the primers given in the table above have proved successful for confirming the presence or absence of pXO1 and/or pXO2 in pure cultures of isolates from animal (including human) specimens or environmental ~~specimens or~~ samples. They ~~are~~ may be unsuitable, however, for direct detection of *B. anthracis* in such specimens or samples. A choice of alternatives can be found in Jackson *et al.* (1998) and Ramisse *et al.* (1996). For the rare possibility that an isolate may lack both pXO1 and pXO2, a chromosomal marker should also be run; primers for these are also ~~supplied~~ described in Jackson *et al.* (1998) and Ramisse *et al.* (1996).

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

1. Background

a) Rationale and intended use of the product

The most widely used vaccine for prevention of anthrax in animals was developed by Sterne (1937). He derived a rough variant of virulent *B. anthracis* from culture on serum agar in an elevated CO_2 atmosphere. This variant, named 34F2, was incapable of forming a capsule and was subsequently found to have lost the pXO2 plasmid, which codes for capsule formation. It has become the most widely used strain world-wide for animal anthrax vaccine production. In Central and Eastern Europe, an equivalent pXO2$^-$ derivative, Strain 55, is the active ingredient of the current livestock vaccine. A list of manufacturers of anthrax vaccine for use in animals is given in ~~Appendix V~~ Annex 5 of WHO (2008).

The following information concerning preparation of the anthrax vaccine for use in animals is based on Misra (1991) and the WHO Expert Committee on Biological Standardization (1967). Generalised procedures are given; national regulatory authorities should be consulted in relation to Standard Operating Procedures that may pertain locally.

~~1. Seed management~~

2. Outline of production and minimum requirements for conventional vaccines

a) Characteristics of the seed

i) Biological characteristics

Anthrax vaccine production is based on the seed-lot system. A seed lot is a quantity of spores of uniform composition processed at one time and maintained for the purpose of vaccine preparation. Each seed lot is no more than three passages from the parent culture and must produce a vaccine that is efficacious and safe for use in animals. It is recommended that a large seed lot be prepared from the parent strain and preserved by lyophilisation for future production lots. The parent culture can be purchased[3].

ii) Quality criteria

The seed lot is acceptable for anthrax vaccine if a vaccine prepared from the seed lot or a suspension harvested from a culture derived from a seed lot meets the requirements for control of final bulk with respect to freedom from bacterial contamination, safety and efficacy (immunogenicity).

b) Method of manufacture

i) Procedure

Preparation of the master seed

2 GE Healthcare http://www.gelifesciences.com ~~Uppsala, Sweden, product number 27-9555-01~~

3 Health Protection Agency Culture Collections, Health Protection Agency, Microbiology Services, Porton Down, Salisbury, SP4 0JG, UK (http://www.hpacultures.org.uk/)

Seed lots are cultured on solid media formulated to promote sporulation of the organism ~~(see Section C.2 below)~~. The solid medium formula for casein digest agar (sporulation agar) given in Misra (1991) is: 50 g tryptic digest of casein; 10 g yeast extract; 0.1 g $CaCl_2.6H_2O$; 0.01 g $FeSO_4.7H_2O$; 0.05 g $MgSO_4.7H_2O$; 0.03 g $MnSO_4.4H_2O$; 5.0 g K_2HPO_4; 1.0 g KH_2PO_4; 22 g agar; 1000 ml deionised or distilled water. The ingredients are dissolved in the water with the appropriate amount of heating; the solution is adjusted to pH 7.4, distributed into Roux bottles (120 ml per bottle) or other appropriate container, sterilised by autoclaving and cooled in the horizontal position. After the agar has solidified, excess liquid should be removed aseptically and the bottles left in an incubator (37°C) for at least 2 days to dry and to check the sterility.

Volumes of 2 ml of vaccine seed ~~from a reference laboratory~~ should be spread across the agar in Roux bottles, which should be incubated at 37°C until at least 80% sporulation is apparent by microscopic examination of aseptically extracted loopfuls (at least 72 hours). The growth is harvested with 10 ml per bottle of sterile deionised or distilled water and checked for purity. After washing three times in sterile deionised or distilled water with final suspension, also in sterile deionised or distilled water, sterilised lyophilisation stabiliser is added and the suspension is dispensed into lyophilisation vials and freeze-dried.

Preparation and testing of the working seed

Reconstitute a vial of seed stock and inoculate several slants (approximately 10 ml) of sporulation (casein digest) agar. Incubate at 37°C for 72 hours and store in a refrigerator. Test the slants for purity by culture on to nutrient agar plates and in nutrient broth (0.1 ml in 100 ml of nutrient broth). The latter should be subcultured on to nutrient agar after incubation at 37°C for 7 days and should be a pure culture of *B. anthracis*. A sample of the broth culture should also be checked for lack of motility.

Volumes of seed needed for a production run should be calculated on the basis of harvesting the spores from each slant with 10 ml of sterile deionised or distilled water and using this to inoculate five Roux bottles.

~~**d) Safety of the seed lot**~~

~~Not less than 5×10^9 culturable spores should be injected subcutaneously into each of three healthy, 1–2 year-old, unvaccinated sheep, which must survive an observation period of at least 10 days.~~

~~**e) Immunogenicity of the seed lot**~~

~~At least 10 healthy guinea pigs, 300–500 g in weight should be inoculated with 5×10^6 viable spores and observed for 21 days. At least 80% of the animals should survive. The immunised animals, together with three unimmunised controls, should then be challenged with 10 median lethal doses (LD_{50}) of the strain 17 JB of *B. anthracis*. During a 10-day observation period, none of the immunised animals should succumb to the challenge while all the controls should die from anthrax. The test should be repeated if one of the immunised animals dies.~~

~~2. Method of production~~

Preparation of vaccine concentrate

Roux bottles with casein digest agar are prepared as for the master seed in Section C.1.b above. One Roux bottle can be expected to yield about 2000 doses of vaccine. Each Roux bottle is inoculated with 2 ml of working seed suspension and incubated at 37°C with porous plugs for several days until small loopfuls of culture from randomly selected bottles show at least 90% of the organisms to be in sporulated forms when examined in wet mounts by phase contrast (phase bright spores) or following staining for spores. The growth from each bottle is then harvested with 20 ml of physiological saline. Tests for contaminants should be carried out by subculture to nutrient agar plates and inoculation of 100 ml nutrient broth with 0.1 ml of harvested spores followed by subculture to nutrient agar after 7 days at 37°C and by tests for motility. Acceptable harvests (i.e. those showing no evidence of contaminants) are pooled.

Glycerination

Twice the volume of sterile, pure, neutral glycerol should be added to the bulk pool of vaccine concentrate. Saponin (0.1% final concentration) may also be added at this point if it is to be included as an adjuvant. Mix thoroughly (the inclusion of sterilised glass beads may be helpful). Carry out a purity test ~~as before~~ and hold for 3 weeks at ambient temperature to allow lysis of any vegetative bacteria, determine the viable spore count and store under refrigeration thereafter.

Determining titre and dilution for use

The number of culturable spores in the product is then calculated by spreading tenfold dilutions on nutrient agar plates. The suspension is diluted so that the final bulk contains the number of culturable spores desired. The diluent should contain the same proportions of saline, glycerol and (if being included) saponin as

present in the vaccine concentrate. The vaccine should contain a minimum of 2–10 × 10^6 culturable spores per dose for cattle, buffaloes and horses, and not less than 1–5 × 10^6 culturable spores per dose for sheep, goats and pigs.

~~**d) Safety**~~

~~Safety testing is performed on two healthy sheep or goats and consists of inoculating subcutaneously twice the recommended vaccination dose. The animals are observed for 10 days. The final bulk passes the test if no systemic reactions develop and if not more than a transient oedema is observed at the injection site. If the test is carried out in sheep only, a progressive oedema indicates that the vaccine may be unsuitable for goats.~~

Filling the containers

Distribution of aliquots of vaccine into single and multidose containers is performed as outlined in WHO Technical Report No. 363 series entitled *General Requirements for Manufacturing Establishments and Control Laboratories* (Requirements for Biological Substances No. 1), 1965, 16–17. Basically, the final bulk is distributed to containers in an aseptic manner in an area not used for production, and any contamination or alteration of the product must be avoided. The vaccine may be lyophilised after distribution into appropriate dosage containers. Containers are sealed as soon as possible with a material that is not detrimental to the product and that is capable of maintaining a hermetic seal for the life of the vaccine.

ii) Requirements for substrates and media

Please refer to Misra (1991) for detailed information on substrates and media used for anthrax vaccine production.

~~3.~~**iii) In-process controls**

Purity of the seed lot

Purity tests consist of microscopic examination of stained smears with culture and motility tests as described in Section C.2.b.

~~*d) Safety of the seed lot*~~

Not less than 5 × 10^9 culturable spores should be injected subcutaneously into each of three healthy, 1–2-year-old, unvaccinated sheep, which must survive an observation period of at least 10 days.

~~*e) Immunogenicity of the seed lot*~~

At least 10 healthy guinea-pigs, 300–500 g in weight should be inoculated with 5 × 10^6 viable spores and observed for 21 days. At least 80% of the animals should survive. The immunised animals, together with three unimmunised controls, should then be challenged with 10 median lethal doses (LD_{50}) of the strain 17 JB of *B. anthracis*. During a 10-day observation period, none of the immunised animals should succumb to the challenge while all the controls should die from anthrax. The test should be repeated if one of the immunised animals dies.

~~**4. Batch control and tests on the final product**~~

iv) Final product batch tests

~~*4.a)*~~*Sterility* *and purity*

The vaccine is a live culture of *B. anthracis* spores; sterility does not apply, but the batches must be tested for freedom from contamination (see Chapter 1.1.9 Tests for sterility and freedom from contamination of biological materials).

Safety

Safety testing is performed on two healthy sheep or goats and consists of inoculating subcutaneously twice the recommended vaccination dose. The animals are observed for 10 days. The final bulk passes the test if no systemic reactions develop and if not more than a transient oedema is observed at the injection site. If the test is carried out in sheep only, a progressive oedema indicates that the vaccine may be unsuitable for goats.

Batch potency ~~*Efficacy*~~

Efficacy or immunogenicity is tested on the final bulk as follows: at least ten healthy 300–500 g guinea-pigs are inoculated with a sheep dose of the vaccine. The guinea-pigs are observed for 21 days, and at least 80% of the animals must survive the observation period. Surviving immunised guinea-pigs and three non-

vaccinated controls are challenged with an appropriate dose of virulent *B. anthracis.* A recommended challenge is 200 LD_{50} of the Pasteur II strain (17JB)~~, which is available from the same source as the Sterne 34F2 vaccine strain~~. If, by 10 days after challenge, all vaccinated guinea-pigs survive and control animals die, the final bulk is deemed to be satisfactory. If any vaccinated animals die during the post-challenge observation period from a cause other than anthrax, and death is not associated with the vaccine, the test may be repeated.

~~**c) Dose**~~

~~The recommended dose for cattle and horses is a minimum of 2–10 × 10^6 culturable spores; for sheep, goats and pigs, it is 1–5 × 10^6 culturable spores. The vaccine should contain these spores in an appropriate volume, e.g. 2 × 10^6/ml.~~

~~**d) Duration of immunity**~~

~~Most experts agree that immunity is good for at least 1 year and it is recommended that an annual booster be given. Horses may be slow to develop immunity following initial vaccination; some manufacturers therefore recommend a two-dose initial vaccination, administered 1 month apart, followed by a single annual booster.~~

~~**e) Stability**~~

~~As there is no generally acceptable test for stability of anthrax vaccines, it is recommended that, in each filling lot, the number of culturable spores be determined before and after holding at an appropriate temperature for an appropriate period. There should be no evidence of a fall in the number of culturable spores.~~

~~**f) Preservatives and storage**~~

~~*Bacillus anthracis* spores are stable in unlyophilised or lyophilised vaccine and preservatives are not required. Storage under refrigeration is recommended (4°C).~~

~~**g) Precautions (hazards)**~~

c) Requirements for authorisation

i) Safety requirements

Target and non-target animal safety

The vaccine has been shown to cause disease in some goats and llamas; this may be related to the saponin adjuvant. The vaccine is not recommended for use in pregnant animals, nor in animals destined for slaughter within 2–3 weeks of vaccination. Local regulations may specify other time periods in some countries or regions, but there is no scientific reason for regarding meat from clinically healthy animals as unfit for human handling or consumption after a holding period of 2 weeks following vaccination. Concurrent administration of antibiotics to vaccinated animals is contraindicated as the antibiotic will interfere with the vaccine. Antibiotics should not be given for several days before and after vaccination. ~~Leftover vaccine, empty vials, and equipment used for vaccinating are contaminated with the live spores and should be autoclaved, disinfected, or incinerated.~~

Accidental human inoculation is treated by expressing as much of the inoculum as possible from the injection site and washing the wound thoroughly with soap and water. Medical attention should be sought if infection develops.

Reversion–to-virulence for attenuated/live vaccines

The 34F2 strain of *Bacillus anthracis* is known to be stable and cannot produce capsule *in vitro*. Attenuated vaccine strains can gradually lose their antigenicity over repeated subculturing conditions. Therefore, it is recommended that master seed lots be made in bulk and kept within three passages from the original seed culture. A large number of master seed stocks should be prepared.

Environmental consideration

Leftover vaccine, empty vials, and equipment used for vaccinating are contaminated with the live spores and should be autoclaved, disinfected, or incinerated.

ii) Efficacy requirements

For animal production

Not applicable.

For control and eradication

The recommended dose for cattle and horses is a minimum of 2–10 × 10^6 culturable spores; for sheep, goats and pigs, it is 1–5 × 10^6 culturable spores. The vaccine should contain these spores in an appropriate volume, e.g. 2 × 10^6/ml. Immunity ~~is~~ should be good for at least 1 year and it is recommended that an annual booster be given. Horses may be slow to develop immunity following initial vaccination; some manufacturers therefore recommend a two-dose initial vaccination, administered 1 month apart, followed by a single annual booster.

Bacillus anthracis spores are stable in unlyophilised or lyophilised vaccine and preservatives are not required. Storage under refrigeration is recommended (4°C).

As there is no generally acceptable test for stability of anthrax vaccines, it is recommended that, in each filling lot, the number of culturable spores be determined before and after holding at an appropriate temperature for an appropriate period. There should be no evidence of a fall in the number of culturable spores.

~~**5. Tests on the final product**~~

~~**a) Safety**~~

~~Every batch of vaccine will be tested for safety as described in Section C.2.d.~~

~~**b) Potency**~~

~~Every batch of vaccine will be tested for potency, as described in Section C.4.b.~~

3. Vaccines based on biotechnology

a) Vaccines available and their advantage

There are no vaccines based on biotechnology available for anthrax.

b) Special requirements for biotechnological vaccines, if any

Not applicable.

~~**6. Propagation of the diagnostic 'gamma' bacteriophage**~~

~~Anthrax-specific phages were first isolated in the 1950s, and the specifically named gamma phage was first reported in 1955 and quickly became the standard diagnostic phage for anthrax. Gamma phage belongs to a family of closely related anthrax phages (13). On occasion a phage-negative *B. anthracis* or phage-positive *B. cereus* is encountered. The phage must be used in conjunction with other tests for confirmation. , yet it is a useful and reliable test.~~

~~Phage suspensions may be obtained from central veterinary laboratories or central public health laboratories.~~

~~The phage can be propagated and concentrated by the following protocol. Store phage at 2–4°C and do not freeze phage as it will quickly become non-viable.~~

~~Stage one~~

~~i) Spread a blood agar (BA) plate of the Sterne vaccine strain of *B. anthracis*. Incubate overnight at 37°C.~~

~~ii) Inoculate approximately 10 ml of nutrient broth (NB) with growth from the BA plate and incubate at 37°C for approximately 4 hours or until just cloudy, then refrigerate.~~

~~iii) Spread 100 µl of the culture from step ii on three pre-dried BA plates and incubate at 37°C for 30–60 minutes.~~

~~iv) Spread 100 µl of the phage suspension to be amplified over the same plates. Incubate at 37°C overnight.~~

~~v) Harvest the phage-lysed growth on the BA plate in 5 ml of NB followed by a second 'wash' of 5 ml NB. Incubate at 37°C overnight.~~

~~vi) Filter (0.45 µm) and count by dropping 20 µl drops (three drops per dilution) of tenfold dilutions of the filtrate in saline onto lawns of the *B. anthracis* culture prepared as in step iii.~~

~~Stage two~~

~~This is essentially the same procedure as Stage one, only using the filtrate from step vi to harvest the phage from the plates.~~

~~vii) Prepare three Sterne strain lawns on BA, as in step iii. Incubate at 37°C for 30–60 minutes.~~

~~viii) Spread 100 µl phage from step vi. Incubate at 37°C overnight.~~

~~ix) To 9 ml of filtrate from step vi, add 1 ml of 10× concentrated NB.~~

~~x) Harvest the phage from step viii with 5 ml of the solution from step ix, followed by a second 5 ml wash with the rest of the solution from step ix.~~

~~xi) Add 10 ml of 1× NB.~~

~~xii) Incubate at 37°C overnight, filter and count.~~

~~Stage three~~

~~xiii) Inoculate 100 ml of brain–heart infusion broth with approximately 2.5 ml of the culture from step ii. Incubate on a rotary shaker at 37°C until just turbid.~~

~~xiv) Add the 20 ml of filtrate from step xii and continue incubation overnight.~~

~~xv) The resultant filtrate is checked for sterility and titrated in ten-fold dilutions on lawns of the vaccine strain as in step vi to determine the concentration of the phage. This should be of the order of 10^8–10^9 plaque forming units per ml.~~

~~7. Polychrome methylene blue (M'Fadyean's stain)~~

~~Polychrome methylene blue is prepared as follows: 0.3 g of methylene blue is dissolved in 30 ml of 95% ethanol; 100 ml of 0.01% potassium hydroxide (KOH) is mixed with the methylene blue solution. Ideally, this should be allowed to stand exposed to the air, with occasional shaking, for at least 1 year to oxidise and mature. Addition of K_2CO_3 (to a final concentration of 1%) hastens the 'ripening' of the stain, but before it is regarded as diagnostically reliable, its efficacy should be established by testing it in parallel with an earlier, functional batch of stain on *bona fide* samples. It has now been found that stains that give positive reactions with cultures of *B. anthracis* cultured artificially in horse blood sometimes do not give positive results in the field.~~

~~In making smears for staining, only small drops of blood or tissue fluid are needed and a thin, small smear is best. After fixing and drying, a small (approximately 20 µl) drop of stain is placed on the smear and spread over it with an inoculating loop. After 1 minute, the stain is washed with water, blotted, air-dried and observed initially using the ×10 objective lens under which the short chains appear like short hairs; once found, these can be observed under oil immersion (×1000) for the presence of the pink capsule surrounding the blue/black-staining bacilli. To avoid laboratory contamination, the slide and blotting paper should be autoclaved or left for some hours in a 10% sodium hypochlorite solution.~~

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NB: There are OIE Reference Laboratories for Anthrax (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).

ADVICE FOR MEMBER COMMENTS

Chapter Number and Title: 2.1.17. Trypanosoma evansi *infection* (*surra*)

Country making the comments

Date

It would be appreciated if the following guidance notes are followed in making a reply:

1. *Comments may be general or specific, but specific comments are usually more valuable. General comments should be such that some conclusion and action can be taken in response to them. For example, it is of little help to make statements such as "This test is no longer used in our laboratory", without indicating the reasons and what test is used instead.*

2. *Specific comments should be identified, by indicating the line number in the text, to facilitate the making of editorial changes.*

3. *The pointing out of typing or technical errors is welcome, but the correct word or figure should be indicated. For example, it is of little help to say that 0.8 M is too high, if the preferred value is not indicated.*

4. *Bear in mind that the introductory chapters are not intended to be exhaustive, and indeed none of the chapters can give a completely comprehensive cover of the subject, otherwise the* Terrestrial Manual *would be too long. However, assistance in indicating priorities is always helpful.*

5. *The* Terrestrial Manual *is intended for world-wide use. The chapters need to reflect the development of new technology, but the old established methods, usually requiring less sophisticated apparatus, have to be retained. New technology should not be described in detail until it has gained wide acceptance as a reliable method.*

General Comments

Specific Comments (*add continuation sheets if required*)
line:

CHAPTER 2.1.17.

TRYPANOSOMA EVANSI INFECTION (SURRA)

SUMMARY

Definition of the disease: Trypanosoma evansi *causes a trypanosomosis known as 'surra'. It affects a large number of wild and domesticated animal species in Africa, Asia, and Central and South America. The principal host species varies geographically, but camels, horses, buffalos and cattle are particularly affected, although other animals, including wildlife, are also susceptible. It is an arthropod-borne disease; several species of haematophagous flies, including* Tabanids *and* Stomoxes, *are implicated in transferring infection from host to host, acting as mechanical vectors. In Brazil, vampire bats are also implicated in a unique type of biological transmission.*

Description of the disease: *The general clinical signs of* T. evansi *infections: pyrexia directly associated with parasitaemia together with a progressive anaemia, loss of condition and lassitude are not sufficiently pathognomonic for diagnosis. Recurrent episodes of fever and parasitaemia occur during the course of the disease. Oedema, particularly of the lower parts of the body, urticarial plaques and petechial haemorrhages of the serous membranes are sometimes observed in horses. Abortions have been reported in buffalos and camels. Nervous signs are common in horses. The disease causes immunodeficiencies that may be of high impact when interfering with other diseases or vaccination campaigns* (*foot and mouth disease and haemorrhagic septicaemia for example*).

Identification of the agent*: The general clinical signs of* T. evansi *infection are not sufficiently pathognomonic for diagnosis. Laboratory methods for detecting the parasite are required. In early infection or acute cases, when the parasitaemia is high, examination of wet blood films, stained blood smears or lymph node materials might reveal the trypanosomes. In more chronic cases, or more generally when the parasitaemia is low, the examination of thick blood smears, as well as methods of parasite concentration and the inoculation of laboratory rodents are required. In apparently healthy carriers* (*animals without clinical signs*)*, parasites are rarely observed and mouse inoculation gives the best results. Several primer pairs targeting the subgenus* (*Trypanozoon*) *or the species-specific* (T. evansi) *parasitic DNA sequences are available for diagnosis by polymerase chain reaction* (*PCR*)*. PCR is more sensitive than parasitological examination, but it may give false-negative results when the parasitaemia is very low; in these cases, suspicion of potential carriers can only be confirmed by serological examination.*

Serological tests: *Infection gives rise to specific antibody responses and a variety of antibody detection tests have been introduced for laboratory and field use. Some have been partially validated, but await large-scale evaluation and standardisation. The most relevant are immunofluorescence test* (*IFAT*)*, enzyme linked immunosorbent assays* (*ELISA*) *and card agglutination test* (*CATT*/T. evansi). *For field use, only CATT*/T. evansi *can be applied. Pen side tests are currently unavailable. Estimates of predictive values indicate that ELISA for detecting IgG is more likely to classify correctly uninfected animals, while the CATT is more likely to classify correctly truly infected animals. ELISA would thus be suitable for verifying the disease-free status of animals prior to movement or during quarantine. CATT can be used to target individual animals for treatment with trypanocidal drugs. For declaring a disease-free status, serial testing – CATT and ELISA followed by re-testing of suspect samples – is recommended, preferably completed by PCR. In areas where* T. cruzi, T. equiperdum *or tsetse-transmitted trypanosomes occur, cross-reactions may occur with any serological test employed.*

Requirements for vaccines: *No vaccines are available for the disease.*

A. INTRODUCTION

Infection with *Trypanosoma evansi* causes a disease named *surra* in India and, amongst others, *El Debab*, *El Gafar,* Tabourit or MBori in North Africa, *Mal de Caderas* or *Murrina* in Latin America. The clinical signs of surra are indicative but are not sufficiently pathognomonic, thus, diagnosis must be confirmed by laboratory methods (Dia *et al.,* 1997a). The disease in susceptible animals, including camels (dromedary and bactrian), horses, buffalo, cattle and pigs is manifested by pyrexia, directly associated with parasitaemia, together with a progressive anaemia, loss of condition and lassitude. Such recurrent episodes lead to intermittent fever (as high as 44°C in horses [Gill, 1977]) and parasitaemia during the course of the disease. Oedema, particularly of the lower parts of the body, rough coat in camels, urticarial plaques and petechial haemorrhages of the serous membranes are sometimes observed. In advanced cases, parasites invade the central nervous system (CNS), which can lead to nervous signs (progressive paralysis of the hind quarters and, exceptionally, paraplegia), especially in horses, but also in other host species before complete recumbency and death. Abortions have been reported in buffalos and camels (Gutierrez *et al*., 2005; Lohr *et al.,* 1986) and there are indications that the disease causes immunodeficiency (Dargantes *et al.,* 2005b; Onah *et al.,* 1998).

There is considerable variation in the pathogenicity of different strains and the susceptibility of different host species. The disease may manifest as an acute or chronic form, and in the latter case may persist for several months, possibly years. The disease is often rapidly fatal in camels and horses, but may also be fatal in buffalo, cattle, llamas and dogs, however these host species may develop mild or subclinical infections. Wild animals such as deer, capybara and coati can become infected and ill (including death), but they may also constitute a reservoir. Animals subjected to stress – malnutrition, pregnancy, work – are more susceptible to disease.

Biologically *T. evansi* is very similar to *T. equiperdum*, the causative agent of dourine (Brun *et al.,* 1998; Claes *et al*., 2003), and morphologically resembles the slender forms of the tsetse-transmitted species, *T. brucei brucei, T. b. gambiense* and *T.b. rhodesiense.* Most of the molecular characterisations indicate that various strains of *T. evansi* isolated from Asia, Africa and South America are very homogeneous and may have a single origin (Ventura *et al.,* 2002), but other works suggest that *T. evansi* could have emerged from *T. brucei* in several instances (Jensen *et al.,* 2008; Lai *et al.,* 2008) Molecular characterisation using random amplified polymorphic DNA techniques and endonuclease fingerprinting showed that isolates of *T. evansi* and *T. equiperdum* formed a closely homogeneous group. The difficulties in differentiating *T. equiperdum* from the other *Trypanozoon* spp. have been stressed (Claes *et al*., 2005; Zablotskij *et al.,* 2003), and the existence of *T. equiperdum* was even questioned.

Like all pathogenic trypanosomes, *T. evansi* is covered by a dense protein layer consisting of a single protein called the variable surface glycoprotein (VSG). This acts as a major immunogen and elicits the formation of specific antibodies. The parasites are able to evade the consequences of these immune reactions by switching the VSG, a phenomenon known as antigenic variation.

Clinical suspicion of surra can emerge from the field in case of fever and/or anaemia. Anaemia is a reliable indicator of trypanosome infection, but it is not in itself pathognomonic. On the other hand, animals with a mild subclinical infection can have parasitaemia without evidence of anaemia (Dargantes *et al.,* 2005a).

In enzootic areas, routine diagnoses can be made using parasitological techniques, while serological surveys can be carried out preferably by ELISA. CATT can be used to target individual animals for treatment with trypanocidal drugs.

For a definitive confirmation of the infection in suspected animals, mouse inoculation is the best test to apply.

For declaring disease-free status, at the individual level, serial testing by CATT and ELISA at 40-day intervals is recommended. However the conditions for importation of animals from infected to non-infected areas should be defined, including the status of the exporting farm, the status of exported animals, the application of a diagnostic protocol, and possibly the preventive administration of curative treatments.

In areas where *T. cruzi, T. equiperdum* or tsetse-transmitted trypanosomoses are present, cross-reactions may occur with any serological test employed. In such conditions, the exact status of an animal regarding trypanosomosis cannot be established.

The OIE has developed international standard monographs for trypanocidal drugs.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

The classical direct parasitological methods for the diagnosis of trypanosomosis, namely microscopic examination of blood or lymph node material, are not highly sensitive, but a number of techniques, including enrichment of the sample, rodent inoculation and DNA methods may increase the sensitivity. In regions where other *Trypanozoon*

spp. occur in addition to *T. evansi*, specific identification by microscopy is not possible; molecular tools are then very useful for species specific diagnosis.

a) Direct microscopic examination

i) *Blood sampling*

Trypanosoma evansi is a parasite of the blood and tissues. As for other trypanosomes, it is recommended that blood for diagnosis be obtained from peripheral ear or tail vein, even if the jugular vein is most often preferred for practical reasons. However it should be realised that less than 50% of infected animals may be identified by examination of blood.

Peripheral blood is obtained by puncturing a small vein in the ear or tail. Deeper samples are taken from a larger vein by syringe. Cleanse an area of the ear margin or tip of the tail with alcohol and, when dry, puncture a vein with a suitable instrument (lancet, needle). Ensure that instruments are sterilised or use disposable instruments to avoid iatrogenic transmission of the infection by residual blood.

ii) *Wet blood films*

Place a small drop of blood (2–3 µl) on to a clean glass slide and place over it a cover-slip to spread the blood as a monolayer of cells. Examine by light microscopy (200 ×) to detect any motile trypanosomes. Improved visualisation can be obtained with dark-ground or phase-contrast microscopy (200–400 ×). The sensitivity of this method is low, approximately 10 trypanosomes per µl, which is frequent in early or acute infections only.

iii) *Stained thick smears*

Place a large drop of blood (10 µl) on the centre of a microscope slide and spread with a toothpick or the corner of another slide so that an area of approximately 1.0–1.25 cm in diameter is covered. Air-dry for 1 hour or longer, while protecting the slide from insects. Placing the slide in a horizontal position, stain the unfixed smear with Giemsa's Stain (one drop of commercial Giemsa + 1 ml of phosphate-buffered saline, pH 7.2), for 25 minutes. After washing and drying, examine the smears by light microscopy at a magnification of 500× with oil immersion. The advantage of the thick smear technique is that it concentrates the drop of blood into a small area, and thus less time is required to detect the parasites, which are more visible owing to the haemolysis of the unfixed red cells. The disadvantage is that the trypanosomes may be damaged in the process, and the method is therefore not suited for species identification in case of mixed infections.

iv) *Stained thin smears*

Place a small drop of blood (3–5 µl) at one end of a clean microscope slide and draw out a thin film in the usual way. Air-dry briefly and fix in methyl alcohol for 1 minute and allow to dry. Stain the smears in Giemsa (one drop Giemsa + 1 ml PBS, pH 7.2) for 25 minutes. Pour off stain and wash the slide in tap water and dry. Nowadays, fast stains are most often used[1], which allow fixation and staining within a few seconds. Slides are then washed in tap water and dried. Examine at a magnification of 400–1000× with oil immersion. This technique permits detailed morphological studies and identification of the *Trypanosoma* species, but it is of a very low sensitivity (it can detect parasitaemia >500,000 trypanosomes/ml of blood).

v) *Lymph node biopsies or oedema fluid*

Samples are usually obtained from the prescapular or precrural (subiliac) lymph nodes. Select a suitable node by palpation and cleanse the site with alcohol. Puncture the node with a suitable gauge needle, and draw lymph node material into a syringe attached to the needle. Expel lymph on to a slide, cover with a cover-slip and examine as for the fresh blood preparations. Fixed thin or thick smears can also be stored for later examination. Similar examination can be done by collection of oedema fluid.

b) Concentration methods

In most hosts *T. evansi* can induce mild clinical or subclinical carrier state infections with low parasitaemia in which it is difficult to demonstrate the parasites. In these circumstances, concentration methods are necessary, as they increase the sensitivity of microscopic examination.

i) *Haematocrit centrifugation technique* (*also known as Woo's technique, or HCT*)

1 For example Diff-Quick®, RAL555®

Collect blood (70 µl) into two heparinised capillary tubes (75 × 1.5 mm). Close the wet end with plasticine and centrifuge at 3000 ***g*** for 5 minutes (generally 12,000 rpm in a haematocrit centrifuge machine). The capillary tube is examined and the value of the haematocrit is expressed as a percentage of packed red blood cells (RBCs) to total blood volume; this gives an indication on the anaemia of the animal. The capillary tube is then placed in a groove made with pieces of slide glued to a slide. Trypanosomes are large cells that concentrate at the junction between the buffy coat and the plasma, which is observed under the microscope (100–200 ×). Light conditions must be set to induce refringence of the cells to increase the visibility of the moving trypanosomes; this can be obtained by lowering the position of the light condenser or with intermediary positions of the turret light condenser. Specially designed reading chambers for HCT can be obtained at the OIE Reference Laboratory for Surra, at the Institute of Tropical Medicine (ITM)[2]. The fresher the sample, the better is the sensitivity as strong parasitic movements make trypanosomes more visible. This technique can detect around 50–200 trypanosomes/ml of blood (Desquesnes & Tresse, 1996). The buffy coat can also be collected in a microtube and frozen; also the sample can be prepared for polymerase chain reaction (PCR).

ii) *Dark-ground/phase-contrast buffy coat method* (*also known as Murray's technique, or BCM*)

This technique is very similar to the previous one. Collect blood into heparinised capillary tubes and centrifuge as above. Scratch the tube with a glass-cutting diamond and break it 0.5 mm below the buffy coat layer – the upper part thus contains a small top layer of RBCs, the buffy coat (white blood cells and platelets) and some plasma.

Partially expel the contents of this piece on to a slide; avoid expelling more than 5–8 µl of plasma, but make sure the buffy coat has been expelled (the small disk of the buffy coat should be visible to the naked eye), press on a cover-slip to spread the buffy coat and examine by dark-ground, phase-contrast or similar microscopy under the previously described refringent conditions at a magnification of 200–500 ×. Trypanosomes are mostly present at the periphery of the thick buffy coat material.

Both the Woo and the Murray techniques allow anaemia to be estimated by measuring the packed cell volume and may be used in surveys of herds at risk. The value of the haematocrit can be used as an indicator (when <24% for example in cattle) to select a subset of samples to be submitted to the more expensive PCR analysis (Desquesnes *et al*., 1999).

iii) *Mini-anion exchange centrifugation technique*

When a blood sample from animals infected with salivarian trypanosomes is passed through an appropriate DEAE-cellulose (diethylamino-ethylcellulose, such as Whatman DE 52) anion-exchange column, the host blood cells, being more negatively charged than trypanosomes, can be adsorbed onto the anion-exchanger (in pH and ionic strength conditions adapted to the host species), while the trypanosomes are eluted, retaining viability and infectivity (Lanham & Godfrey, 1970). This technique is mostly used for the purification of parasites from the blood (for example, for parasite antigen preparation), but miniature systems have been developed, especially for diagnosis in humans (Lumdsen *et al.,* 1981). A simplified field method for detection of low parasitaemia has been developed (Sachs, 1984). The sensitivity of this technique can be increased by approximately tenfold by the use of buffy coat preparations rather than whole blood (Reid *et al.,* 2001).

- *Preparation of phosphate buffered saline glucose* (*PSG*), *pH 8*

Na_2HPO_4 anhydrous (13.48 g); $NaH_2PO_4.2H_2O$ (0.78 g); NaCl (4.25 g); distilled water (1 litre). Solutions of different ionic strength are made by diluting the stock PBS, pH 8, and adding glucose to maintain a suitable concentration. For blood of mice, domestic and wild ruminants and dog, add four parts PBS to six parts distilled water and adjust the final glucose concentration to 1%. For blood of pigs and rabbits, add three parts PBS to seven parts distilled water and adjust the final glucose concentration to 1.5%. The PBS/glucose solution (PSG) must be sterile (however, PBS must be autoclaved before adding glucose).

- *Equilibration of DEAE-cellulose*

Suspend 500 g of DEAE-cellulose in 2 litres of distilled water. Mix for 20 minutes with a magnetic stirrer at low speed. Adjust the pH to 8 with phosphoric acid. Allow to settle for 30 minutes. Discard the supernatant fluid containing the finest granules. Repeat the procedure three times. Store the equilibrated concentrated suspension of DEAE-cellulose (slurry) at 4°C for a short period, or in small aliquots at –20°C for longer conservation.

- *Packing of equilibrated DEAE-cellulose*

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Place a 2 ml syringe without the plunger on a test-tube rack complete with a flexible tube that can be closed with a clamp to act as a tap. Put a disc of Whatman No. 41 filter paper at the bottom of the syringe and moisten by adding a few drops of PSG. Pour 2–2.5 ml of the slurry of equilibrated cellulose into the syringe and allow packing for 5 minutes before elution of the buffer. The height of the sediment should be approximately 3 cm. Wash and equilibrate the column with 2 ml of PSG without disturbing the surface.

- *Adsorption of blood eluate of the trypanosomes*

Gently place 100–300 µl of heparinised blood (or preferably buffy coat) on the surface of the cellulose column; allow it penetrate the cellulose, but do not let the cellulose dry before pouring on the eluting buffer. Progressively add 1.5 ml of PSG and start collecting the eluate into a finely tapered Pasteur pipette with a sealed end. The cellulose column should remain wet throughout the procedure. Put the filled pipette, protected by a conical plastic pipette tip, in a tube and centrifuge at 525 ***g*** (or up to 1000 ***g***) for 10 minutes. Examine the bottom of the pipette under the microscope (100 × or 200 ×) using a special mounting device. Alternatively, the eluate could be collected into 50 ml plastic tubes, with conical bottoms, centrifuged at 1000 ***g*** and the sediment examined by dark-ground microscopy.

A similar method used in cattle, pig and goat is also referred to as the miniature anion exchange chromatography method (Gutierrez *et al*., 2004a; Reid *et al.,* 2001; Sachs, 1984). In addition, large amounts of blood or buffy coat can be applied to large columns for preparation of antigen for indirect fluorescence antibody test (IFAT), card agglutination test (CATT) or enzyme linked immunosorbent assay (ELISA).

c) Animal inoculation

Laboratory animals may be used to reveal subclinical (non-patent) infections in domesticated animals. *Trypanosoma evansi* has a broad spectrum of infectivity for small rodents, and so rats and mice are often used. Rodent inoculation is not 100% sensitive (Monzon *et al.,* 1990) but further improvement in its efficacy can be obtained by the use of buffy coat material. Such a procedure was able to detect as few as 1.25 *T. evansi*/ml blood (Reid *et al.,* 2001). This technique is suitable when highly sensitive detection is required.

Inoculate heparinised blood intraperitoneally into rats (1–2 ml) or mice (0.25–0.5 ml). Inoculate a minimum of two animals. Bleed animals from the tail after every 48 hours to detect parasitaemia. The incubation period before appearance of the parasites and their virulence depends on the strain of trypanosomes, their concentration in the inoculum, and the strain of laboratory animal used; however in most cases it is very short (5 ± 2 days), but can extend to 2 weeks in rare cases (Monzon *et al.,* 1990). Sensitivity of this *in vivo* culture system may be increased by use of immunosuppressed laboratory animals. Drugs such as cyclophosphamide or hydrocortisone acetate, X-ray irradiation, or splenectomy have been used for this purpose. Such a procedure is only justified when the detection of a potentially infected host is of high importance (for example, importation into a disease-free area).

d) Detection of trypanosomal DNA

Detection of minute amounts of trypanosomal DNA is a possible mean of identifying animals with active infections as the parasitic DNA does not remain for more than 24–48 hours in the blood of the host after the trypanosomes are killed (Desquesnes, 1997b).

- **DNA probes**

Specific DNA probes have been used to detect trypanosome DNA in infected blood or tissue but are not routinely applied as further evaluation needs to be made (Basagoudanavar *et al.,* 2001; Reid *et al.,* 2001; Viseshakul & Panyim, 1990). PCR techniques are generally preferred and are routinely used in some laboratories.

- **Polymerase chain reaction (PCR)**

Polymerase chain reaction (PCR) based on DNA sequences of various taxonomic levels is used. The gold standard, to date, for detection of the *Trypanozoon* subgenus are the NRP or TBR primers (Masiga *et al.,* 1992; Moser *et al.,* 1989). Other primers have been published and are being evaluated; some of them are specific for *Trypanozoon* (Desquesnes *et al*., 2001; Holland *et al*., 2001; Wuyts *et al.,* 1994) and others for *T. evansi* ± *T. equiperdum* (Artama *et al.,* 1992; Claes *et al*., 2004; Panyim *et al.,* 1993) (evaluation of the latter is very difficult because of the absence of collections of reference strains). To date, the most sensitive test is that of satellite DNA using TBR primers (Masiga *et al.,* 1992); the sensitivity of the other primers is being compared under various conditions, including in laboratory rodents, but can only be validated with a sufficient batch of field samples from natural hosts. The use of TBR primers is recommended, at least in the first instance, and, if necessary, for example in areas and host species potentially infected with other *Trypanozoon* such as *T. brucei brucei*, species confirmation can be obtained with more specific primers such as TEPAN (Panyim *et al.,* 1993) or TE2249/2250 (Artama *et al.,* 1992). Other primers specific for RoTat

(Claes *et al*., 2004; Verloo *et al.,* 2001) or non-RoTat strains (Ngaira *et al.,* 2005), and other techniques such as the loop-mediated isothermal amplification (LAMP) (Thekisoe *et al.,* 2005) and Taqman (Taylor *et al.,* 2008), are under development but need to be further evaluated and validated.

DNA preparation is an important step that determines the success and the sensitivity of the PCR. It can be done on plain blood (generally collected with anticoagulant), or, preferably, on the buffy coat to increase the sensitivity of the test (Desquesnes & Davila, 2002; Majiwa *et al.,* 1994). Several classical techniques are available, such as Chelex preparation (Solano *et al.,* 1999), commercial kits and the phenol–chloroform preparation (Maciel *et al.,* 2009). Blood conserved 1/1 in 70% alcohol, or on dry filter paper can also be used (Desquesnes, 2004; Holland *et al*., 2002; Omanwar *et al.,* 1999).

The sensitivity of the PCR being dependent on the amount of DNA available, it is proportional to the parasitaemia. PCR is thus more sensitive in highly susceptible hosts (camels, horses, dogs, etc.) than in hosts of mild or low susceptibility (cattle, buffalo, pigs, etc.). Using a suitable DNA preparation and the most sensitive primers available (TBR), PCR allows as little as 1–5 trypanosomes/ml of blood to be detected (Panyim *et al.,* 1993; Penchenier *et al.,* 1996), or only 10 per ml in buffaloes with a quantitative real-time PCR (Konnai *et al.,* 2009).

PCR offers the sensitivity and specificity required for detection of trypanosome infection (Masiga *et al.,* 1992; Wuyts *et al.,* 1994; Wuyts *et al.,* 1995), but it may give false-negative results. Experimental studies in sheep have shown that PCR can remain negative for long intervals during aparasitaemic periods (Bengaly *et al*., 2001), while in buffalo the diagnostic sensitivity of a PCR was only 78%, which is similar to mouse inoculation (Holland *et al*., 2001). Nevertheless, PCR is the most sensitive technique for detection of the infection.

f) Antigen detection

Circulating antigen detection in blood or serum is also a way to detect active infection. Several attempts to develop such tests have not yet reached a satisfactory level to be recommended for routine diagnosis (Desquesnes, 1996; Monzon, 2006; Morzaria *et al.,* 1996).

2. Serological tests

Historically, different methods have been used to detect non-specific humoral antibodies present in cases of surra infection. These methods are biochemical tests including flocculation, formol-gel, mercuric chloride precipitation and thymol turbidity tests, and are considered to be outdated although the formol-test may still have some use in the field because it is simple to perform. These tests all depend on an increase in serum globulins as a result of infection, but this increase is not specific for *T. evansi* infection. Mercuric chloride must not be used because of its toxicity. The formol-gel test is the test of choice in camels but has not been validated in other species. It is carried out by adding two drops of concentrated formalin solution (40% formaldehyde [w/v]) to 1 ml of serum. The test is positive if the serum coagulates immediately and turns white. In negative reactions, the serum remains unchanged or coagulation may take up to 30 minutes to appear.

Similarly, many different methods have been used to detect specific humoral antibodies to trypanosomal antigens, such as direct or indirect agglutination tests, complement fixation test (CFT), IFAT (Desquesnes, 1997a; Uilenberg, 1998) and the trypanolysis test. The IFAT is still useful for small-scale surveys. The trypanolysis test is used for individual confirmation of positivity because of its high specificity. The other tests are no longer used as, recently, they have been replaced by the more easily standardised techniques of ELISA (Davison *et al.,* 1999; Franke *et al.,* 1994; Rae *et al.*, 1989; Reid & Copeman, 2002; Reid & Copeman, 2003; Tuntasuvan *et al.,* 1996) and CATT (Bajyana Songa & Hamers, 1988; Njiru *et al*., 2004). Attempts to develop new techniques such as latex agglutination tests have not been successful so far (Gutierrez *et al*., 2004b; Holland *et al*., 2005; Morzaria *et al.,* 1996).

Evaluations of ELISA and CATT have been carried out in camels, horses, cattle, buffaloes and pigs (Desquesnes *et al.,* 2009; Diall *et al.*, 1994; Holland *et al*., 2005; Payne *et al.* 1991; Reid & Copeman, 2003; Verloo *et al.,* 2000). Tests should preferably be carried out on plasma or serum, but the collection of samples can be simplified by using filter paper blood spots for later use in the ELISA, while for the CATT whole blood can be substituted for serum (Holland *et al*., 2002; Hopkins *et al.,* 1998). It is vitally important that serological tests be validated and standardised if they are to be suitable for correctly identifying infected animals; cross evaluation in different laboratories is thus required. Standard criteria for interpreting the tests might have to be developed for each animal species and standardised at least at a regional level (Desquesnes, 1997c). It is also necessary to take into consideration the various *Trypanosoma* species and strains (RoTat *versus* non-RoTat for example) present in a given area.

a) Indirect immunofluorescent antibody test (IFAT)

Although the technique is not adapted to large-scale surveys, it is still useful to screen a small number of samples in laboratories that are carrying out the test for other purposes and/or that are not carrying out the ELISA. Cost of reagents is medium, around 0.5€/test, but the technique is time consuming.

- **Test procedure**

The antigen consists of dried blood smears containing from five to ten *T. evansi* per field at magnification 500×, collected from a highly parasitaemic mouse or rat (3–4 days post-infection). Smears are dried at room temperature for 1 hour and fixed with acetone (± ethanol) for 5 minutes. When kept dry, the fixed smears may be stored at –20°C for several months. Better results are obtained using purified trypanosomes separated from the rat's buffy coat on a DEAE-cellulose column (Lanham & Godfrey, 1970) using a mixture of 80% cold acetone and 0.25% formalin in a normal saline solution.

On testing, the slides are first subdivided into several circles of 5 mm diameter with nail varnish using mounting media (Teflon-coated multispot slides may also be used), then washed in PBS, pH 7.2, at room temperature for 10 minutes.

After washing, a positive and a negative control serum and field sera to be tested (diluted 1/50 in PBS), are added and allowed to react at 37°C for 30 minutes in a humid chamber. The slides are washed three successive times in PBS for 5 minutes each. A rabbit or goat anti-bovine IgG (for tests on bovine sera) conjugated to fluorescein isothiocyanate or other fluorescein-conjugated antiserum specific to the animal species tested is then added at a suitable dilution and left at 37°C for 30 minutes in a humid chamber. The slides are rewashed in PBS, mounted with 50% glycerol in PBS with immunofluroescence mounting media, and examined by fluorescence microscopy. The glycerol solution should be stored at 4°C and renewed every 2 weeks.

The fluorescein conjugate should be stored at –20°C in small aliquots to avoid repeated freezing and thawing. The tube should be shielded from light in some way, for example by wrapping in aluminium foil. The conjugate is diluted in PBS, pH 7.2, or in PBS containing Evans blue 1/1000 (w/v) as a counterstain to facilitate discrimination between positive (green) and negative (red) fluorescence. In general, monospecific anti-IgG (gamma-chain) conjugates give the most specific results.

The IFAT-*T. evansi* seroconversion can take 60–90 days (Jacquiet *et al.*, 1993). Compared with the CATT, IFAT is more sensitive, probably because it can detect aparasitaemic animals, but specificity is lower (Dia *et al.,* 1997b). In borderline cases, the interpretation is subjective and reproducibility has sometimes been questioned (Ferenc *et al.,* 1990). For these reasons, ELISA is a more advisable technique.

b) Enzyme-linked immunosorbent assay (ELISA)

The principle of this technique is that specific antibodies to trypanosomes can be detected by enzyme-linked anti-immunoglobulins using solid-phase polystyrene plates coated with soluble antigen. The enzyme may be peroxidase, alkaline phosphatase or any other suitable enzyme. The enzyme conjugate binds to the antigen/antibody complex and then reacts with a suitable substrate to yield a characteristic colour change either of the substrate itself or of an added indicator (the chromogen).

The antigen for coating the plates is derived from the blood of a heavily parasitaemic rat. The trypanosomes are concentrated in the buffy coat by centrifugation and separated on a DEAE-cellulose column and washed three times by centrifugation in cold PSG, pH 8 (PBS with 1% glucose). The final pellet is suspended in cold PSG to a concentration of 5%, together with a protease inhibitor cocktail[3] subjected to five freeze–thawing cycles, and ultrasonicated three times for 2 minutes on ice to ensure complete disintegration of the organisms. This preparation is centrifuged at 4°C and 14,000 ***g*** for 10 minutes. The supernatant is collected and the protein concentration estimated by UV readings at 260 and 280 nm (Warburg & Christian, 1942). The soluble antigen thus obtained can be stored in small aliquots at –80°C for several months. It can also be freeze-dried and stored at –20°C. Coating of the ELISA plate is generally made with 5 µg/ml protein concentration in coating buffer.

- **Test procedure**

i) Dilute the soluble antigen at 5 µg/ml in freshly prepared 0.01 M carbonate/bicarbonate buffer, pH 9.6. Add 100 µl to each well of a 96-well microtitre plate and incubate overnight at 4°C or for 1 hour at 37°C. For this step immunoplates that ensure that the specific activities of the epitopes are preserved during

3 Complete solution for protease inhibitor; Roche Molecular Biochemicals

binding to the plate surface[4], are preferred to other plates that may allow epitopes to be obscured or impaired due to the binding characteristics .

ii) Remove antigen and add 150 µl of blocking buffer (BB: 0.01 M PBS containing 0.1% Tween 20 and 5% skim milk powder for 1 hour at 37°C. The quality of the skim milk is very critical[5]; optimal skim milk concentration may vary from 0.5 to 7% depending on the skim milk origin. Bovine serum albumin may also be used as blocking agent.

iii) Add test serum dilutions in BB (100 µl), in duplicate or triplicate. Include control negative and positive sera. Dilutions must be determined empirically, but are usually around 1/100–1/200. Incubate plates at 37°C for 30 minutes. Eject contents and wash five times with washing buffer (PBS-0.1% Tween 20).

iv) Add a specific peroxidase conjugated species-specific anti-globulin (100 µl) appropriately diluted in BB (usually between 1/5000 and 1/20,000). If species-specific conjugates are not available, protein A or protein G conjugates can be used. Incubate the plates at 37°C for 30 minutes, eject contents and wash three times with washing buffer.

v) For peroxidase conjugates a number of substrate/chromogen solutions can be used, consisting of hydrogen peroxide with a chromogen, such as tetramethylbenzidene (TMB), 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and ortho-diphenylenediamine (OPD). A suitable substrate/chromogen solution for peroxidase conjugates is 30% hydrogen peroxide (0.167 ml and 35 mg) in citrate buffer (100 ml), pH 6.0. The citrate buffer is made up as follows: Solution A (36.85 ml): (0.1 M citric acid [21.01 g/litre]); Solution B (65.15 ml): (0.2 M, Na_2HPO_4 [35.59 g/litre]); and distilled water (100 ml). Dissolve 10 mg TMB in 1 ml dimethyl sulphoxide and add to 99 ml of the citrate buffer. A number of these combinations are available commercially in ready-to-use formulations that remain stable at 4°C for up to 1 year. Add the substrate chromogen (100 µl) to the plates and incubate at room temperature for 20–30 minutes.

vi) Read the plates or stop the reaction by adding 50 µl 1 M sulphuric acid. Read the absorbance of each well at 450 nm for TMB chromogen. Other chromogens may require the use of a different wavelength. All tests should include three known high and medium positive control sera, three low and medium negative control sera, and a buffer control. Results are expressed in relative percentage of positivity based on the optical densities of the control samples (Desquesnes, 1997c; Desquesnes *et al.,* 2009).

A large variety of other test procedures exists, for example, using purified native antigen (Verloo *et al.,* 1998) or, more recently, using recombinant antigens (Tran *et al.,* 2009). For closely related animal species, cross-reacting reagents may often be used (e.g. anti-bovine immunoglobulin for buffalos) and the use of monospecific anti-IgG conjugates is generally recommended. However, when specific conjugates are not available, nonspecific proteins able to fix on the Fc fragment of the immunoglobulins can be used, such as protein A (for detection of IgG) or protein G (for detection of IgM). Protein A conjugate has been validated for use in camels (Desquesnes *et al*., 2009).

There is a number of methods that can be used to determine a cut-off point to discriminate between positive and negative results. The simplest method is to base the cut-off on visual inspection of the test results from known positive and negative populations (Desquesnes, 1997c). These results are likely to show some overlap. The operator can choose the most appropriate point to modify the false-positive or false-negative results depending on the required application of the assay. An alternative is to base the cut-off on the mean + 2 standard deviations (SD) or + 3 SD values from a large sample of negative animals. Finally, if no suitable negative/positive samples are available, a cut-off can be based on the analysis of the data from animals in an endemic situation (Greiner *et al*., 1994). If a bimodal distribution separates infected from uninfected animals, then an appropriate value can be selected. The ELISA is likely to correctly identify uninfected animals (while the CATT would correctly qualify infected ones). A new ELISA/RoTat 1.2 based on the VSG from a *T. evansi* RoTat 1.2 clone – a predominant antigen in *T. evansi* (Verloo *et al.,* 2001) – was successfully used in the field in Vietnam (Holland *et al*., 2002; Verloo *et al.,* 2000); protocols are available from the OIE Reference Laboratory at ITM (Antwerp) for use in equines, camelidae and water buffaloes. Another test based on invariant surface glycoprotein has recently been developed at the ITM (Tran *et al.,* 2009) and should proceed to inter-laboratory evaluation.

The VSGs may be too specific to be used as antigen in a universal test (see below RoTat *versus* non-RoTat parasites), while the ELISA using soluble antigens is not strain specific and this qualifies it as a universal test. Soluble antigens from whole lysate of *T. evansi* are able to detect immunoglobulins directed against *T. evansi* strains present in various host species and geographical areas (Laha & Sasmal, 2008); they can also detect infections in heterologous systems owing to strong cross reactions with *T. vivax*, *T. congolense* and even *T. cruzi. Trypanosoma evansi* soluble antigen must then be considered as a universal reagent for detection of *T. evansi*, but consideration must be given to species specificity in multispecies areas. The cost

4 For example Polysorp Nunc® immunoplates
5 For example: ref: 190-12865, Wako Pure Chemical Industries Ltd, Osaka, Japan.

of reagents is low, around 0.1€/test, and the technique is fast, allowing 500–1000 samples to be tested a day by experienced technicians.

c) Card agglutination tests

It is well known that certain predominant variable antigen types (VATs) are expressed in common in different strains of salivarian trypanosomes from different areas. This finding was used as a basis for a test for the diagnosis of *T. evansi*, the card agglutination test – CATT/*T.evansi.* The test makes use of fixed and stained trypanosomes of a defined VAT known as RoTat 1.2. Both variable and invariable surface antigens take part in the agglutination reaction. The CATT is available in kit form from the OIE Reference Laboratory ITM. It consists of lyophilised stained parasites ('antigen'), PBS, pH 7.4, plastic-coated cards, spatulas, positive and negative control sera and a rotator. The lyophilised antigen can be stored at 2–8°C for up to 1 year. Reconstituted antigen can be stored at 2–8°C for 1 week, but preferably should be used within 8 hours when kept at 37°C.

For screening, dilute test sera 1/4 or 1/8 in PBS on to circles inscribed on the plastic cards. Add 45 µl of the prepared antigen suspension (previously well shaken to homogenise the parasite suspension) onto circles inscribed on the plastic cards. Add 25 µl of each test serum. Mix and spread the reagents with a spatula and rotate the card for 5 minutes using the rotator provided in the kit (or at 70 rpm on a classical rotor agitator). Compare the pattern of agglutination with the illustrations of different reactions provided in the kit. Blue granular deposits reveal a positive reaction visible to the naked eye. The cost of reagents is medium, around 0.5€/test, around 200 tests can be carried out a day by one technician.

As the CATT principally detects IgM (agglutinating pentavalent immunoglobulins the half life of which is short), it is suitable for detection of early infections or late infections with recent circulation of parasites in the blood, and can detect active infections with a high positive predictive value. The CATT is more likely to classify correctly truly infected animals, it can be used to target individual animals for treatment with trypanocidal drugs.

An alternative test format (LATEX/*T.evansi*) using latex beads coated with native RoTat 1.2 VSG is currently under evaluation.

d) Immune trypanolysis test

Immune trypanolysis test detects specific 'trypanolytic' antibodies directed against a given parasitic strain able to induce trypanolysis in the presence of complement. It is performed with *T. evansi* variable antigen type RoTat 1.2 and may therefore be positive only with hosts that produce trypanolytic immunoglobulins directed against RoTat 1.2 VAT (Van Meirvenne *et al.,* 1995). Sera are tested at a 1/4 dilution. Live trypanosomes are incubated for 60 minutes with test serum in the presence of guinea-pig serum as the source of complement. When variant specific antibodies are present in the serum, lysis of the RoTat 1.2 trypanosomes occurs. The sample is considered positive for the presence of anti-RoTat 1.2 antibodies when 50% or more of the trypanosomes are lysed. This test requires the growth of trypanosomes in rodents and is thus costly. At present, it is mostly used to confirm samples suspected to be positive using other tests. It can be carried out at the ITM, Antwerp, on request. The cost of the test is very high (250€/test).

3. Test applications

Like the majority of biological tests, the methods described above are limited both in terms of sensitivity and specificity. Moreover, test performances and parameters are highly variable, from a host species or from one geographical area to another. To date, there is no common test (parasitological, serological and even molecular one) that allows distinguishing *T. evansi* from the other *Trypanozoon* species or sub-species. The final diagnosis of surra will come out from epizootiological information and laboratory results and observations. For these reasons, a number of test combinations adapted to each circumstance and for the most concerned host species are currently recommend. These are guidelines that should be helpful in achieving the correct diagnoses.

a) Recommended methods

i) *Microscopic examination:* Microscopic observation (×400–1000 in oil immersion) of a Giemsa-stained thin blood smear from the host, or from a mouse inoculation test, allows identification of the subgenus *Trypanozoon* based on morphology and morphometry of parasites. When fresh samples are available, HCT or BCM must be used to increase the sensitivity.

ii) PCR-TBR: DNA must be prepared by blood commercial kit or phenol chloroform method, from a buffy coat obtained by 8000 ***g*** centrifugation of 0.5 ml of blood. PCR is carried out as described above, with TBR primers (Masiga *et al.,* 1992). Result is positive for *Trypanozoon* when a 177 bp product is visible on the agarose gel.

iii) ELISA *T. evansi:* Serum or plasma samples are tested in ELISA-*T. evansi* (soluble antigens from whole *T. evansi* lysate) as described above. A sample is positive when its RPP is > to the cut off line established for the host species (a conjugate is defined for each species; see (d) below).

iv) CATT/*T. evansi:* Serum or plasma are diluted 1:4 and tested as described by manufacturer. Positive samples are samples presenting results = or > to one + (doubtful samples are considered as negative samples).

b) Equines

An equine is negative to surra tests if it is negative to ELISA-*T. evansi* (anti-horse IgG whole molecule), CATT/*T. evansi*, PCR-TBR and microscopic examination.

An animal is considered as infected by a *Trypanozoon* if it is positive to PCR-TBR and/or if *Trypanozoon* parasites are observed by microscopic examination.

An animal is considered as seropositive to surra tests if it is positive to ELISA-*T. evansi* and/or CATT/*T. evansi*; In this case, the animal should be tested for CFT-dourine, and, if it is positive for CFT-dourine it is also considered as seropositive to dourine; if it is negative to CFT-dourine, it is considered as seropositive to surra only.

c) Camelids

An animal is negative to surra tests if it is negative to ELISA-*T. evansi* (protein A conjugate), CATT/*T. evansi*, PCR-TBR and microscopic examination.

An animal is considered as infected by a *Trypanozoon* if it is positive to PCR-TBR and/or microscopic examination.

An animal is considered as seropositive to surra tests if it is positive to ELISA-*T. evansi* and/or CATT/*T. evansi*.

d) Other host species

An animal is negative to surra tests if it is negative to ELISA-*T. evansi* (conjugate see below), CATT/*T. evansi*, PCR-TBR and microscopic examination.

An animal is considered as infected by a *Trypanozoon* if it is positive to PCR-TBR and/or microscopic examination.

An animal is considered as seropositive to surra tests if it is positive to ELISA-*T. evansi* and/or CATT/*T. evansi*.

Conjugates to be used for each host species: Cattle and buffalo: anti-bovine IgG whole molecule; Pig and elephant: Protein G conjugate; Camels: Protein A conjugate; Rat: anti-rat IgG whole molecule. Conjugates remind to be defined for other host species.

C. REQUIREMENTS FOR VACCINES

No vaccines are available for this disease. Fulfil

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ADVICE FOR MEMBER COMMENTS

Chapter Number and Title: 2.3.1. Avian chlamydiosis

Country making the comments

Date

It would be appreciated if the following guidance notes are followed in making a reply:

1. *Comments may be general or specific, but specific comments are usually more valuable. General comments should be such that some conclusion and action can be taken in response to them. For example, it is of little help to make statements such as "This test is no longer used in our laboratory", without indicating the reasons and what test is used instead.*

2. *Specific comments should be identified, by indicating the line number in the text, to facilitate the making of editorial changes.*

3. *The pointing out of typing or technical errors is welcome, but the correct word or figure should be indicated. For example, it is of little help to say that 0.8 M is too high, if the preferred value is not indicated.*

4. *Bear in mind that the introductory chapters are not intended to be exhaustive, and indeed none of the chapters can give a completely comprehensive cover of the subject, otherwise the* Terrestrial Manual *would be too long. However, assistance in indicating priorities is always helpful.*

5. *The* Terrestrial Manual *is intended for world-wide use. The chapters need to reflect the development of new technology, but the old established methods, usually requiring less sophisticated apparatus, have to be retained. New technology should not be described in detail until it has gained wide acceptance as a reliable method.*

General Comments

Specific Comments (*add continuation sheets if required*)

line:

SECTION 2.3.

AVES

CHAPTER 2.3.1.

AVIAN CHLAMYDIOSIS

SUMMARY

Avian chlamydiosis (*AC*) *is caused by the bacterium* ~~Chlamydophila~~ Chlamydia psittaci*. AC occurring in humans and all birds was originally called psittacosis, but later the term ornithosis was introduced to identify the disease contracted from or occurring in domestic and wildfowl, while the name of the disease contracted from or occurring in psittacine birds remained psittacosis. These diseases are similar when contracted by humans. The* ~~*genus* Chlamydia *was recently divided into two*~~ *former genera* Chlamydia *and* Chlamydophila *have recently been re-combined into the genus* Chlamydia ~~*All known avian strains are now in the species* Chlamydophila psittaci*. Chlamydiosis is still the term used for diseases produced by both genera*~~*. The avian strains of* Chlamydia psittaci *include at least* ~~*six serotypes that*~~ *fifteen genotypes, some of which correlate with the avian species from which they are usually isolated. Chlamydiosis as it occurs naturally in mammalian species and not contracted from avian species is caused by* ~~*distinctly*~~ *different* ~~*strains of the organism*~~ *species of* Chlamydia*.*

Depending on the virulence of the chlamydial ~~*serovar*~~ *strain and the avian host defence, chlamydiae cause pericarditis, conjunctivitis, sinusitis, airsacculitis, pneumonia, lateral nasal adenitis, peritonitis, hepatitis, and splenitis. Generalised infections result in fever, anorexia, lethargy, diarrhoea, and occasionally shock and death. Special laboratory handling is (biosafety level 3) recommended because avian chlamydial strains can cause serious illness and possibly death in humans. While the disease in psittacine birds is best known, the infection* ~~*The disease*~~ *in ducks and turkeys is of particular concern as transmission to humans is common during handling and slaughter of the birds. The diagnosis of AC requires the isolation and identification of the organism, the demonstration of chlamydiae in tissues, or the demonstration of a four-fold increase in specific humoral antibody, as well as typical clinical signs.*

Identification of the agent: *Isolation of chlamydiae requires the inoculation of embryonated eggs or cell cultures and testing for chlamydiae by cytochemical stains or immunohistochemical methods. The direct inoculation of samples into cell cultures is preferable as they are as sensitive for the isolation of most avian strains of chlamydiae as are chicken embryos. The cell cultures are then stained by* ~~*direct*~~ *immunofluorescence or by other appropriate stains at appropriate times to demonstrate the presence of inclusions.*

Histochemical staining of impression smears from the liver, heart, and spleen are commonly made. The technique gives a rapid diagnosis, but requires some experience.

Enzyme-linked immunosorbent assays (*ELISAs*) *developed for detecting* Chlamydia *trachomatis antigen in humans have been used for diagnosing chlamydiae in birds. Many of the earlier tests were developed using monoclonal or polyclonal antisera against lipopolysaccharide epitopes, some of which were shared with other Gram-negative bacteria. Their use when screening individual birds is questionable, as they lack sensitivity and specificity.*

Molecular tools (*polymerase chain reaction restriction length polymorphism, DNA microarray or sequencing*) *and immunohistochemical staining of histological sections are* ~~*new techniques showing promise for the future*~~ *now widely used in diagnostic laboratories. All of them are rapid and do not*

require the live agent. The current PCR tests target the ompA ~~MOMP~~ gene or the ribosomal RNA genes (16S or 23S) ~~and some will amplify all chlamydial strains and allow identification at the level of the chlamydial species~~. Validated and standardised protocols of both species-specific and family-specific assays are available. Nested and real-time PCRs can be as sensitive as isolation. There has been an increase in the use of immunohistochemical staining of histological sections because of the recent development and availability of automated staining equipment.

***Serological tests:** The standard serological test for chlamydial antibodies is the complement fixation (CF) test. The modified direct CF test can be used with most sera. The antigen is a group-reactive lipopolysaccharide antigen present in all strains. The occurrence of high CF titres in the majority of individuals in a flock with clinical signs is presumptive evidence of active infection. The demonstration of a four-fold increase in titre in an individual bird is considered to be diagnostic of a current infection.*

Other serological tests, such as the ELISA, latex agglutination, elementary body agglutination, micro-immunofluorescence, and the agar gel immunodiffusion tests ~~are available~~ can be used. These tests are of value in specific cases and may replace the CF test; however, comparisons of reliability and reproducibility are not yet available.

***Requirements for vaccines ~~and diagnostic biologicals~~:** There are no commercial vaccines available for chlamydiosis control in poultry. Antibiotics are the only current means of control. ~~Chlamydophila~~ Chlamydia psittaci is susceptible to a number of antibiotics. The drug of choice varies from country to country.*

A. INTRODUCTION

Avian chlamydiosis (AC) is caused by the bacterium *Chlamydia* (formerly *Chlamydophila*) *psittaci*. The disease in birds was originally called psittacosis, but the term ornithosis was introduced later to differentiate the disease in domestic and wild fowl from the disease in psittacine birds. The two syndromes are currently considered to be the same (Andersen & Vanrompay, 2003). Their earlier separation was based on the assumption that in humans, ornithosis was a milder disease than psittacosis. However, it should be noted that the disease in humans contracted from turkeys and ducks is often as severe as that contracted from psittacine birds.

~~*Chlamydophila psittaci* produces a~~ Infection of birds with *Chlamydia psittaci* is common all over the world and has been found in about 465 avian species (Kaleta & Taday, 2003). Outbreaks of AC in psittacine birds and domestic poultry farms cause considerable economic damage (European Commission, 2002).The infection can lead to systemic and occasionally fatal disease in birds. The clinical signs are generally nonspecific and vary greatly in severity, depending on the species and age of the bird and the strain of chlamydia. AC can produce lethargy, hyperthermia, abnormal excretions, nasal and eye discharges, and reduced egg production. Mortality rates will vary greatly. In pet birds the most frequent clinical signs are conjunctivitis, anorexia and weight loss, diarrhoea, yellowish droppings, sinusitis, biliverdinuria, nasal discharge, sneezing, lachrymation and respiratory distress (Mohan, 1984). Many birds, especially older psittacine birds, may show no clinical signs; nevertheless, they may often shed the agent for extended periods. Necropsy of affected birds will often reveal multifocal hepatic necrosis, spleen and liver enlargement, fibrinous airsacculitis, pericarditis and peritonitis (Andersen & Vanrompay, 2003; Vanrompay *et al.,* 1995). Histological lesions are suggestive of infection but are non-pathognomonic unless there are identifiable chlamydiae present.

The taxonomy of the family *Chlamydiaceae* was again revised recently ~~reclassified~~ by abolishing the subdivision into the ~~two~~ genera ~~and nine species based on sequence analysis of its 16S and 23S rRNA genes (13). The two new genera,~~ *Chlamydia* (*C.*) and *Chlamydophila* ~~correlate with the former species *Chlamydia trachomatis* and *C. psittaci*. The~~ that had been introduced in 1999 (Everett *et al.,* 1999a). Now the recombined genus *Chlamydia* (Kuo *et al.,* 2011) includes all nine species, i.e. *C. trachomatis* (human), *C. suis* (swine), *C. muridarum* (mouse, hamster), ~~The genus *Chlamydophila* includes~~ *C. psittaci* (birds and others), *C. felis* (cats), *C. abortus* (sheep, goats, cattle), *C. caviae* (guinea-pigs), and ~~the former species~~ *C. pecorum* (sheep, cattle) and *C. pneumonia* (human and others). While most of these organisms are highly host specific, *C. pneumonia* and *C. psittaci* have a broader host range. The latter has been reported to occur not only in birds and humans, but also in cattle, sheep, swine, horses and other animals (Sachse *et al.,* 2009a).

~~The two genera and nine species have merit both molecularly and for classification of host range and clinical disease. The species show a high degree of correlation with host range, disease syndrome, and virulence, and thus provide an understanding of the epidemiology of the various species and serovars affecting livestock and birds. The terms 'chlamydiosis' and 'chlamydia(e)' are used as generic terms to refer to members of either and both genera. However, the new scientific names are used when referring to a specific chlamydial species.~~

~~The avian strains all belong to the species *Chlamydophila psittaci*. This species includes six known avian serovars and two mammalian serovars, M56 from muskrats and WC from cattle (13). M56 and WC were each isolated from a single outbreak. The six avian serovars are labelled A through F and each shows host specificity. The hosts that each serovar has been associated with are: A, psittacine birds; B, pigeons; C, ducks and geese; D, turkeys; E, pigeons and ratites; and F is a single isolate from a psittacine bird. What is not known is how many of these birds and mammals are the natural hosts of the serovars.~~

The avian strains associated with AC belong to the species *Chlamydia psittaci.* Until recently, nine different genotypes based on the ompA gene coding for the major outer membrane protein (MOMP) were distinguished. Seven of these "classical serovars" are thought to predominantly occur in a particular order or class of Aves and two in non-avian hosts, i.e. genotype A in psittacine birds, B in pigeons, C in ducks and geese, D in turkeys, E in pigeons, ducks and others, E/B in ducks, turkeys and pigeons, F in parakeets, WC in cattle, and M56 in rodents. Most of the avian genotypes have also been identified sporadically in isolates from cases of zoonotic transmission to humans, particularly A, B and E/B (Gaede *et al.,* 2008; Heddema *et al.,* 2006; Vanrompay *et al.,* 2007). Meanwhile, subgroups for three of the more heterogeneous genotypes have been introduced, i.e. A-VS1, A-6BC, A-8455, EB-E30, EB-859, EB-KKCP, D-NJ1, D-9N, and six new provisional genotypes to cover the strains that were previously non-typable have been suggested, i.e. 1V, 6N, Mat116, R54, YP84, and CPX0308, thus bringing the total number of genotypes to 15 (Sachse *et al.,* 2008).

Recent evidence suggests that *Chlamydia psittaci* is not the only chlamydial agent occurring in birds (Gaede *et al.,* 2008; Laroucau *et al.,* 2009). The taxonomic classification of these new agents within or outside the family *Chlamydiaceae* has yet to be defined and their epidemiological importance is still unclear. They seem to be quite widespread in ducks, chickens and pigeons, and some strains appear to act as facultative pathogens. It is important to use diagnostic methods that are capable of differentiating between these organisms and *C. psittaci.*

Antibiotics are the only current means of control. ~~*Chlamydophila*~~ *Chlamydia psittaci* is susceptible to a number of antibiotics: the drug of choice varies from country to country. Chlortetracycline, doxycycline, and other tetracyclines are the most commonly used. Treatment needs to be maintained for extended periods of time. For pet birds, 45 days is often recommended (Smith *et al.,* 2005; Vanrompay *et al.,* 1995).

The strains of avian chlamydiae can infect humans and should be handled carefully, at biosafety level 3 ~~under conditions of biocontainment~~ (Smith *et al.,* 2005). Most infections occur through inhalation of infectious aerosols. While the disease in psittacine birds is best known, the infection in ducks and turkeys is of particular concern as transmission to humans is common during handling and slaughter of the birds (Dickx *et al.,* 2010). Post-mortem examinations of infected birds and handling of cultures should be done in laminar flow hoods or with proper protective equipment. Human infection can result from transient exposures. The incubation period is usually 5–14 days; however, longer incubation periods are known. Human infections vary from inapparent to severe systemic disease with interstitial pneumonia and encephalitis. The disease is rarely fatal in properly treated patients; therefore, awareness of the danger and early diagnosis are important. Infected humans typically develop headache, chills, malaise and myalgia, with or without signs of respiratory involvement. Pulmonary involvement is common; auscultatory findings, however, may appear to be normal or to underestimate the extent of involvement. Diagnosis can be difficult and is usually established through testing paired sera for antibodies to chlamydia by the complement fixation (CF) test. In humans, tetracycline, doxycycline, or azithromycin are usually the drugs of choice unless contraindicated. The length of treatment will vary with the drug, but should be continued for at least 14 days for tetracycline.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

The preferred method for the identification of AC is the isolation and identification of the organism. Because of the time involved, the need for high-quality samples, and the hazard to laboratory personnel, other techniques are often used. These include histochemical staining of smears of exudate and faeces, and impression smears of tissues, immunohistochemical staining of cytological and histological preparations, antigen-capture enzyme-linked immunosorbent assays (ELISA), conventional and real-time polymerase chain reaction (PCR), PCR-RFLP (restriction fragment length polymorphism), DNA microarray-based detection and ~~identification systems or~~ DNA sequencing.

a) Collection and treatment of samples for isolation

The samples to be collected will depend on the disease signs in evidence. They must be taken aseptically. Contaminant bacteria may interfere with the isolation of the chlamydiae. Specimens from acute cases should include inflammatory or fibrinous exudate in or around organs that display lesions, ocular and nasal exudates, impression smears of liver, whole blood and tissue samples from kidney, lung, pericardium, spleen, and liver. In cases with diarrhoea, colon contents or excrement should be cultured. In live birds, the preferred samples

are pharyngeal and nasal swabs (Andersen, 1996). Intestinal excrement, cloacal swabs, conjunctival scrapings, and peritoneal exudate can also be taken.

Proper handling of clinical samples is necessary to prevent loss of infectivity of chlamydiae during shipping and storage. A special medium consisting of sucrose/phosphate/glutamate (SPG) was developed for rickettsiae and has proven to be satisfactory for transport of chlamydial field samples. The medium as recommended for chlamydiae (Spencer & Johnson, 1983) consists of SPG buffer: sucrose (74.6 g/litre); KH_2PO_4 (0.512 g/litre); K_2HPO_4 (1.237 g/litre); and L-glutamic acid (0.721 g/litre), which can be sterilised by autoclaving or filtering. Added to this are fetal calf serum (10%), vancomycin, kanamycin, and streptomycin (200–500 µg/ml), amphotericin B and gentamicin (50 µg/ml). The addition of antibiotics reduces the effect of contamination, even when samples are shipped at ambient temperatures. This medium can also be used as a laboratory diluent and for freezing of chlamydiae.

Contaminated samples must be pre-treated before being used to inoculate animals or cell cultures. There are three basic methods: treatment with antibiotics (Bevan *et al.,* 1978), treatment with antibiotics together with low-speed centrifugation (Andersen & Vanrompay, 2003; 2005), and treatment with antibiotics with filtration (Andersen & Vanrompay, 2005; Bevan *et al.,* 1978). A number of antibiotics that do not inhibit chlamydia can be used. Samples are homogenised in phosphate buffered saline (PBS), pH 7.2, containing a maximum of the following: streptomycin (1 mg/ml), vancomycin (1 mg/ml), and kanamycin (1 mg/ml). Gentamicin (200 µg/ml) can be used. Amphotericin B (50 µg/ml) can be added to control yeast and fungal growth. ~~Other antibiotic solutions are often used~~. Penicillin, tetracycline and chloramphenicol should be avoided as these inhibit the growth of chlamydiae.

When contamination is light, samples should be homogenised in an antibiotic solution prior to inoculation into chicken embryos or tissue cultures. Samples are often left to stand in the antibiotic solution for 24 hours at 5°C before inoculation. Heavily contaminated samples, such as faecal samples, should be homogenised in antibiotics and then centrifuged at 500 g for 20 minutes. The surface layer and the bottom layer are discarded. The supernatant fluid is collected and recentrifuged. The final supernatant fluid is used for inoculation. Samples should be passed through a filter of 450–800 µm average pore size if contamination persists.

b) Isolation in cell culture

Cell cultures are the most convenient method for the isolation of *C. psittaci*. The most common cell lines are buffalo green monkey (BGM), McCoy, HeLa, African green monkey kidney (Vero), and L cells (Vanrompay *et al.,* 1992). The cells are grown as monolayers using standard tissue culture media containing 5–10% fetal calf serum and antibiotics that are not inhibitory to chlamydia (as described previously).

When selecting cell culture equipment, it is important to remember that:

i) Chlamydiae can be identified by direct or indirect immunofluorescence or some other appropriate staining technique;

ii) The inoculum is usually centrifuged on to the monolayer to enhance its infectivity;

iii) The sample may need to be blind passaged at 4–5 days to increase sensitivity of isolation;

iv) The sample will need to be examined from two to three times during any one passage; and

v) Chlamydiae can be infectious to humans.

Small flat-bottomed vials, such as 1-dram (3.7 ml, 15 × 45 mm) shell vials or bottles containing cover-slips that are 12 mm in diameter, will meet these requirements (Bevan *et al.,* 1978). A number of vials, often four to six, are inoculated with each sample to permit fixing and staining at various intervals, and to permit repassaging of apparently negative samples 6 days after inoculation. When testing multiple samples, 96-well multiwell dishes can also be used as they have a labour-saving advantage. However, it should be noted that cross-contamination between samples can be a problem.

Chlamydiae can be isolated from cells that are replicating normally, but the use of non-replicating cells is preferable as these may provide increased nutrients for the growth of chlamydiae. Suppressed cells can also be observed for longer periods. Host cell division can be suppressed either by irradiation or, more commonly, by cytotoxic chemicals. The latter include 5-iodo-2-deoxyuridine, cyto-cholasin B, cycloheximide, and emetine hydrochloride. Cycloheximide is the most commonly used and can be added to the medium at the rate of 0.5–2.0 µg/ml at the time of inoculation of the monolayer (Andersen & Vanrompay, 2003; 2005; Bevan *et al.,* 1978). Emetine is removed after treatment and replaced by medium. The monolayer is first treated for 5 minutes with emetine (0.5 µg/ml), after which the emetine is removed and replaced with culture medium; the monolayer is then ready for use. The growth of most chlamydial strains will be enhanced by the treatment of the monolayer by one of these drugs~~; the treatment will have little or no effect on the growth of other strains~~.

Attachment of chlamydia to cells is increased by centrifuging the inoculum on to the monolayer at 500–1500 ***g*** for 30–90 minutes at 37°C. The inoculum is removed and replaced with tissue culture medium containing a cell-division inhibitor, and then incubated at 37–39°C. Cultures must be examined for chlamydiae at regular intervals using an appropriate staining method. This is usually done on day 2 or 3, as well as on day 4 or 5. Cultures that appear to be negative at the fifth day are harvested and repassaged. When repassaging chlamydiae, cells and culture media should be passaged without using freeze–thawing to disrupt cells, as this will destroy the chlamydiae.

Before staining the cultures, the medium is first removed, the cultures are washed with PBS and fixed with acetone for 2–10 minutes. The fixation time will depend on the tissue culture vessel used. As acetone will soften most plastics, the use of a mixture of 50% acetone and 50% methyl alcohol may be preferable. A number of staining methods can be employed to demonstrate chlamydial inclusions. The preferred method is direct immunofluorescence (Andersen & Vanrompay, 2005; Bevan *et al.,* 1978; Moore & Petrak, 1985). A chlamydial fluorescein-conjugated antiserum is applied to the infected cells and incubated in a humid chamber for 30 minutes at 37°C. The cover-slips are then washed three times with PBS, air-dried, mounted, and examined. Chlamydial inclusions fluoresce a green colour. Commercial conjugate preparations using monoclonal antibodies (MAbs) are available and are highly specific. Conjugates may also be prepared from polyclonal sera, but it is important to obtain specific, high-titred antisera. Polyclonal antisera can be prepared in rabbits, guinea-pigs, sheep or goats. Sheep and goats are excellent sources because of the volume and high titres that are readily obtained following infection. Conjugates are then prepared using standard techniques (Andersen & Vanrompay, 2003; 2005).

Chlamydial inclusions can also be demonstrated by indirect fluorescent antibody and immunoperoxidase techniques (Andersen & Vanrompay, 2005; Page, 1974). Direct staining can be done with Gimenez, Giemsa, Ziehl–Neelsen, or Macchiavello's stains. Except for immunofluorescence, all these stains have the advantage that standard light microscopes can be used.

c) Isolation in eggs

Chicken embryos are still used for the primary isolation of chlamydiae. The standard procedure is to inject up to 0.5 ml of inoculum into the yolk sac of a specific pathogen free 6–7-day-old embryo (Andersen & Vanrompay, 2003; 2005). The eggs are then incubated in a humid atmosphere at 39°C, rather than at 37°C, as multiplication of chlamydiae is greatly increased at the higher temperature. Replication of the organism usually causes the death of the embryo within 3–10 days. If no deaths occur, two additional blind passages are usually made before designating any sample as negative. Chlamydial infections will give rise to a typical vascular congestion of the yolk sac membranes. These are harvested and homogenised as a 20% (w/v) suspension in SPG buffer, and can be frozen to preserve the strain, or inoculated into eggs or on to cell cultures.

The organism can be identified by preparing an antigen from an infected yolk sac and testing it by direct staining of smears using appropriate stains or by using the antigen in a serological test. Cell culture monolayers can be inoculated with the yolk sac suspension and examined by direct immunofluorescence 48–72 hours later for the presence of chlamydial inclusions. Typical inclusions are intracytoplasmic round or hat-shaped bodies. With some virulent strains, the inclusions rapidly break up and the chlamydial antigen is dispersed throughout the cytoplasm.

d) Differentiating among species/strains

Chlamydia psittaci can be identified using PCR-RFLP (Everett & Andersen, 1999) or species-specific conventional PCR (Messmer *et al.,* 1997; Sachse & Hotzel, 2003; Van Loock *et al.,* 2005), or real-time PCR (Everett *et al.,* 1999b; Geens *et al.,* 2005; Pantchev *et al.,* 2009; reviewed in Sachse *et al.,* 2009b). A DNA microarray assay was shown to differentiate among all nine species of the family *Chlamydiaceae* (Sachse *et al.,* 2005).

Serotyping in its classical form (Andersen, 1991; 1997) is only rarely conducted because the serovar-specific monoclonal antibodies are not available from a commercial supplier. Instead, genotyping is a practical alternative because i) the classical genotypes A-F are equivalent to the corresponding serotypes, and ii) nine of the more recently defined genotypes cannot be characterised by serotyping because of the absence of specific antibodies. Genotypes A-F can be identified using PCR-RFLP (Vanrompay *et al.,* 1997). A DNA microarray procedure, which was shown to differentiate among all currently known genotypes (Sachse *et al.,* 2008; 2009a), can be used for genotyping of *C. psittaci* strains from cell culture and tissue samples. In addition, all genotypes can be identified by complete sequencing of the *omp*A gene.

As mentioned above, *C. psittaci* is not the only chlamydial agent encountered in birds. The recently described new chlamydial agents (Gaede *et al.,* 2008; Laroucau *et al.,* 2009) have to be taken into consideration, when a given avian sample is positive in a general chlamydial test, e.g. *Chlamydiaceae*-specific PCR or immunohistochemistry, but negative in a species-specific test for *Chlamydia psittaci*. In such a case, partial or complete sequencing of the *omp*A gene and the 16–23S rRNA operon will reveal the

identity of the strain. The occurrence of *Chlamydia abortus* in birds is rare event, but should also be considered as a possible differential diagnosis.

~~All avian isolates are in the *Chlamydophila psittaci* group, as discussed earlier (13). The avian strains can be differentiated from other chlamydiae by PCR-RFLP of either the MOMP gene or the 16S–23S rDNA operon (12). A DNA microarray technology was recently developed in order to differentiate chlamydiae strains (29). A provisional *C. psittaci* determination can be made using the source of the isolate and serovar-specific MAbs.~~

~~The avian strains of *C. psittaci* contain at least six serotypes determined by serovar-specific MAbs (1, 3, 6). The syndromes caused by the various strains are quite specific; the natural host range of a particular strain may also be fairly specific. Serotypes are labelled A through F. The hosts from which they are mainly isolated are: serotype A, psittacine birds; serotype B, pigeons; serotype C, ducks; serotype D, turkeys; serotype E, pigeons and ratites; and one isolate of serotype F from a psittacine bird.~~

~~Avian strains could be differentiated by molecular tools like PCR-RFLP (3, 30, 37). Serotyping and PCR-RFLP have been compared (33, 35) and sometimes incongruent results have been observed. A new genotype, named E/B, was recently identified; sequencing clearly demonstrated that new genotypes cannot always be discovered by PCR-RFLAP or serotyping.~~

e) Histochemical staining

Giemsa, Gimenez, Ziehl–Neelsen and Macchiavello's stains are commonly used to detect chlamydiae in impression smears of liver and spleen. The following modified Gimenez technique is used by several laboratories (Andersen & Vanrompay, 2005):

- **Modified Gimenez technique or (Pierce-van der Kamp) stain**
- *Reagents:*

  Solution 1: Distilled H_2O (450.0 ml) and phenol (5.0 ml) added to basic fuchsin (2.5 g) and 95% ethanol (50.0 ml). Incubate at 37°C for 48 hours. Filter and store in the dark at room temperature.

  Solution 2: Na_2HPO_4 (11.65 g); $Na_2HPO_4.H_2O$ (2.47 g); distilled H_2O, pH 7.5 (to 1.0 litre).

  Solution 3: Solution 1 (20.0 ml); and solution 2 (25.0 ml). Let stand for 10 minutes, filter and use.

  Solution 4: 0.5% citric acid.

  Solution 5: Fast green (0.2 g); distilled H_2O (100.0 ml); and glacial acetic acid (0.2 ml).

  Solution 6: Solution 5 (20.0 ml); and distilled H_2O (50.0 ml).

- *Procedure for smears is as follows:*

i) Fix in methanol for 5 minutes.

ii) Stain in Solution 3 for 10 minutes and rinse in tap water.

iii) Counterstain in Solution 6 for 2 minutes.

iv) Rinse in tap water and air-dry.

- *Procedure for paraffin sections is as follows:*

i) Deparaffinise and hydrate with distilled H_2O.

ii) Stain in Solution 3 for 10 minutes and rinse in tap water.

iii) Dip in Solution 4 until no more red runs out of the section. Rinse in tap water.

iv) Counterstain in Solution 6 for 20 dips.

v) Dip in two changes of 95% alcohol, for five dips each. Dehydrate, clear, and mount.

Chlamydiae will appear red against a green background.

f) Immunohistochemical staining

Immunohistochemical staining can be used to detect chlamydiae in cytological and histological preparations. The technique is more sensitive than histochemical staining, but some experience is necessary as cross-reactions with some bacteria and fungi require that morphology must be considered.

Most widely used immunohistochemical staining procedures can be adapted to give satisfactory results. The selection of the primary antibody is very important. Both polyclonal and monoclonal antibodies have been

used. Because formalin affects chlamydial antigens, it is recommended that polyclonal antibodies be made to purified formalin-inactivated chlamydiae. The chlamydial strain used is not important, as the antibodies will be mainly to the group-reactive antigens. MAbs should also be selected for reactions to formalin-fixed chlamydiae. A pool of group-reactive MAbs can be used.

g) Enzyme-linked immunosorbent assays

The ELISA ~~is a relatively new technique~~ that has been extensively promoted ~~as kits~~ in kit format for use in the diagnosis of human chlamydiosis. These test kits detect the lipopolysaccharide (LPS) antigen (group reactive) and will detect all species of *Chlamydiaceae*. A number of these kits have been tested for use in detecting chlamydiae in birds (Vanrompay *et al.,* 1994), but none of the kits has been licensed for detection of *C. psittaci*. One problem with some of these tests is that the chlamydial LPS shares some epitopes with other Gram-negative bacteria, and these epitopes can cross-react, resulting in a high number of false-positive results. This problem has been reduced or eliminated in more recently developed kits by careful selection of the MAbs used. These kits, however, still lack sensitivity because a few hundred organisms are still needed to give a positive reaction. Most diagnosticians believe that a diagnosis of AC can be made when a strong positive ELISA reaction is obtained from birds with signs of psittacosis. Because of the number of false-positive results, a positive in an individual bird without signs of disease is not considered to be significant, but indicates the need for further testing using different methods.

h) DNA detection systems ~~Polymerase chain reaction and PCR-based systems~~

- **Polymerase chain reaction**

PCR techniques have been ~~are~~ replacing isolation for the detection of chlamydiae ~~in animals~~ from animal tissue. Infection risks to laboratory staff are avoided by inactivation of the sample prior to testing. The sensitivity and specificity ~~can equal or~~ will usually exceed that of isolation ~~and the sample can be inactivated prior to testing~~. Current PCR tests for detection of *C. psittaci* target the ompA ~~MOMP~~ gene or the 16S–23S rRNA gene (Everett *et al.,* 1999b; Geens *et al.,* 2005; Messmer *et al.,* 1997; Pantchev *et al.,* 2009; Sachse & Hotzel, 2003; Van Loock *et al.,* 2005; and reviewed in Sachse *et al.,* 2009b). The sensitivity and specificity varies on sample preparation and the PCR test. Reagents designed to stabilise the DNA should be considered when a delay in processing the sample is anticipated (DeGraves *et al.,* 2003). DNA samples can be prepared using inexpensive reagents or using commercially available kits (Andersen & Vanrompay, 2005). Sensitivity is increased by targeting a relatively short DNA segment, using a nested procedure or using real-time PCR techniques. The nested PCR can equal isolation in sensitivity and specificity (Messmer *et al.,* 1997; Sachse & Hotzel, 2003; Van Loock *et al.,* 2005). However, the risk of contamination is increased if extreme care is not taken when manipulating the reactions (see Chapter 1.1.4/5 Principles and methods of validation of diagnostic assays for infectious diseases). ~~Some excellent nested procedures are available (23, 28, 36). The~~ In the last few years, real-time PCR has become the preferred method in diagnostic laboratories for its rapidity, high throughput and ease of standardisation (Sachse *et al.,* 2009b). This technology requires a fluorescent-labelled probe and special equipment, which increases costs. ~~The~~ Its sensitivity can be equivalent to ~~approach~~ that of the nested system, but contamination problems and labour are reduced as it is based on ~~uses~~ one reaction in a closed system (Everett *et al.,* 1999b; Geens *et al.,* 2005; Pantchev *et al.,* 2009). ~~Targeting the 16S–23S gene also increases sensitivity as multiple copies are usually present in the organism; however, cross-reactions with other bacteria can be a problem. Sequencing of PCR products will allow comparison with the sequences of reference avian chlamydia isolates and the sequence can be used in phylogenic analysis for classification and epidemiological purposes.~~

- **Real-time PCR procedure (Pantchev *et al.*, 2009)**

This assay is conducted as a duplex amplification that includes an internal amplification control (IAC). A detection limit of 2 inclusion-forming units per reaction mix was determined for this assay.

i) The *C. psittaci*-specific oligonucleotides are primers CppsOMP1-F (5'-CAC-TAT-GTG-GGA-AGG-TGC-TTC-A-3') and CppsOMP1-R (5'-CTG-CGC-GGA-TGC-TAA-TGG-3'), as well as MGB® probe CppsOMP1-S (FAM-CGC-TAC-TTG-GTG-TGA-C-TAMRA). The IAC system includes primers EGFP1-F (GAC-CAC-TAC-CAG-CAG-AAC-AC) and EGFP10-R (CTT-GTA-CAG-CTC-GTC-CAT-GC), as well as TaqMan probe EGFP-HEX (HEX-AGC-ACC-CAG-TCC-GCC-CTG-AGC-A-BHQ1). Plasmid IC2 serves as the ICA template[1].

ii) The amplification is conducted in 96-well microtitre plates on an Mx3000P thermocycler or comparable equipment. Each 25-µl reaction contains 12.5 µl of 2 × TaqMan Universal PCR Master Mix supplemented with ROX[2] or a comparable product. The final concentration is 0.9 µM for each *C. psittaci* primer, 0.3 µM for each IAC primer, and 0.2 µM for each probe.

1 Available from Intype IC-DNA, Labordiagnostik Leipzig, Germany.
2 Available from Applied Biosystems.

iii) IAC template DNA (0.5 µl containing 10^4 copies) is added to each reaction before the final volume is made up with water.

iv) The following cycling parameters are used: initial heating cycle at 95°C for 10 minutes (single denaturation step), 45 cycles of 95°C for 15 seconds and 60°C for 1 minute (annealing and extension).

v) The cycle threshold value (Ct) automatically calculated by the software should be used. Ct values higher than 36 should be treated with caution as they may represent cross-reaction with related microorganisms. In such cases, the respective samples should be re-examined by an alternative test.

- **DNA microarray**

DNA microarray technology was recently ~~developed in order to detect chlamydiae (29).~~ shown to be a powerful tool in the diagnosis of chlamydial infections (Sachse *et al.,* 2005). The assay ~~has also proved suitable for unambiguous species identification of Chlamydia in cell cultures and has demonstrated a potential for direct detection of these bacteria from clinical tissues~~ for detection and identification of *Chlamydiaceae* spp. includes identification of *C. psittaci*. It has been validated and proved suitable for routine diagnosis (Borel *et al.,* 2008). Its sensitivity is comparable to that of real-time PCR and specificity is even higher because sample DNA is hybridised to 36 specific oligonucleotide probes. This methodological approach enables detection of mixed chlamydial infections and identification of unexpected chlamydial species directly from clinical samples.

2. Serological tests

a) Modified direct complement fixation test for *Chlamydia*

The following is a widely used modified direct CF test for the detection of antibody. The reagents are relatively easy to prepare and standardise. There are other CF tests; each has advantages. The modified direct CF test is performed in 96-well round-bottom multiwell dishes. Incubation steps are usually done by floating the plates in a 37°C water bath. The chlamydial antigen can be prepared from either infected yolk sacs or cell culture preparations. The modified direct CF test differs from the direct CF test in that normal, unheated chicken serum from chickens without chlamydial antibody is added to the complement dilution. The normal serum increases the sensitivity of the CF procedure so that it can be used to test sera from avian species whose antibodies do not normally fix guinea-pig complement.

- **Test procedure**

i) *Dilution of sera*

Figure 1 gives a suggested pattern for performing the test in round-bottom, 96-well multiwell dishes. All sera must be heat-inactivated at 60°C for 30 minutes prior to use. The sera are diluted in Veronal (barbiturate) buffer saline (VBS) as shown in Figure 1. The dilutions are made in the multiwell dish by adding 100 µl of VBS to each well of rows A and E, and then adding 25 µl of the undiluted sera, positive serum, or negative serum to each of three wells. This gives a starting dilution of 1/5. Then, 25 µl of VBS is added to each well in row B through to D and row F through to H. Twofold dilutions are made, using a 25 µl micropipette, from row A through to D and row E through to H. Appropriate volumes are discarded from the starting and finishing rows to give 25 µl per well. Diluters are rinsed twice in distilled H_2O and once in VBS between each serum.

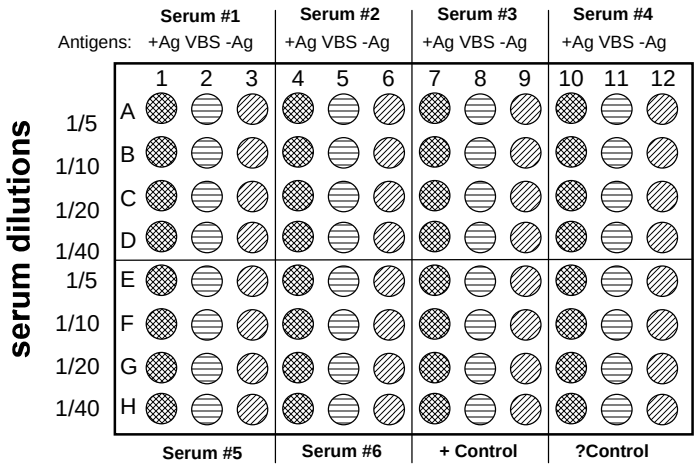

***Fig. 1**. Suggested test pattern for the modified direct complement fixation test when using 96-well dishes.*

ii) *Addition of antigen*

To each well in columns 1, 4, 7, and 10, add 25 µl of positive chlamydial antigen. In columns 2, 5, 8, and 11, add 25 µl of VBS (anticomplementary control wells), and in columns 3, 6, 9, and 12, add 25 µl of negative antigen (normal yolk sac or cell culture prepared the same as the chlamydial antigen). The chlamydial antigens are stored undiluted at 4°C and diluted to proper concentration in VBS prior to use.

iii) *Addition of complement*

Complement (C') is stored at –70°C and should be thawed and diluted in VBS prior to the addition of the antigen. Fresh chicken serum is added before diluting the C' to give a 5% concentration in the complement. Dilutions of C' are made as in previous tests or from titrations. C' should be allowed to stand in an ice bath to stabilise for 15 minutes. The diluted C' should be stored at 4°C following stabilisation and should be used within 2 hours: 50 µl of the C' is added to each well immediately following the addition of the antigens. The plates are incubated uncovered in a 37°C water bath for 2 hours.

iv) *Addition of sheep red blood cells*

Mix 4% standardised sheep red blood cells (SRBCs) with an equal volume of VBS. To this add an equal volume of haemolysin diluted in VBS. The final dilution is incubated in a 37°C water bath for 15 minutes to sensitise the SRBCs. To each well add 50 µl of sensitised SRBCs. The plates are then incubated for 1 hour in a 37°C water bath. The plates can be centrifuged at 400 ***g*** for 5 minutes before reading or they can be refrigerated at 4°C overnight prior to reading.

v) *Interpretation of the results*

The wells are often scored 1+, 2+, 3+, or 4+ corresponding to reduction of haemolysis of 25, 50, 75, or 100%. A positive reaction is 2+ or higher, which is equivalent to 50% or less lysis of the SRBCs. This indicates that the C' was fixed by antibody prior to the addition of the SRBCs. Negative wells are indicated by the complete lysis of the cells: the C' remains unbound and reacts with the SRBCs and the haemolysin to produce lysis of the SRBCs.

Invalid tests occur when the serum is anticomplementary and a positive reaction occurs in the dilution with VBS as the antigen. Nonspecific serum reactions give positive reactions in both the positive and negative wells.

- **Reagents**

i) *Antigen preparation*

The simplest methods start with the growth of chlamydiae in cell culture. The two methods described below produce antigens that can be used in the micro-CF test. The procedures are quite similar: both include the growth of chlamydiae in cell culture, the inactivation of the chlamydiae, partial purification of the antigen, mechanical disruption, and dilution into the appropriate buffer. The method selected will depend on the equipment available.

The first procedure (Grimes, 1985; Grimes *et al.,* 1970) starts with the chlamydiae and cell culture debris harvested when cytopathic effects are noted. The culture is inactivated by the addition of phenol to a final concentration of 1.0%, incubated for 24 hours at 37°C, and concentrated by centrifugation at 10,000 ***g*** for 1 hour. The sediment is reconstituted to 10% of the original volume using VBS, pH 7.2, containing 1.0% phenol and 1.0% glycerol.

The sediment is then homogenised in an omnimixer at top speed for three 1-minute periods while cooled in ice water. The homogenate is centrifuged for 15 minutes at 100 ***g*** to remove debris. Some procedures suggest heating the antigen for 30 minutes in a boiling water bath at this time. The supernatant is saved and diluted to the desired concentration.

In the second procedure for the production of antigen for the CF test (Bracewell & Bevan, 1986), antigen is prepared from L cells infected with a psittacine strain. The cell culture medium is discarded, and the cells are heated for 40 minutes at 56°C. The cells are lysed in distilled water, the chlamydiae are disrupted by ultrasonication, and then made isotonic in VBS. The antigen is tested against a standard sheep convalescent serum and used at 2 units in the micro-CF test.

There are a number of procedures for preparing the antigen from infected yolk sacs, some of which are quite elaborate. However, with the following procedure it is relatively easy to prepare a crude infected yolk sac antigen that works well in the modified direct CF test. An egg-adapted strain of chlamydia is used to inoculate 6–7-day-old embryonated chicken eggs via the yolk sac. The yolk sacs are harvested from embryos that die between 3 and 7 days post-inoculation. The yolk-sac harvest is diluted 1/3 in PBS, Tris buffer, or cell culture medium, and then autoclaved for 20 minutes. The suspension is cooled and then homogenised thoroughly. The use of a high-speed tissue homogeniser for 3–5 minutes is

recommended. After homogenisation, phenol is added to make a final concentration of 0.5% phenol (prepare a 5% phenol stock solution and add 1 ml for every 9 ml of antigen). The antigen preparation is prepared, held for 3 days, and then the supernatant is used after centrifugation for 20 minutes at 1000 ***g***. The antigen can be stored for long periods of time at 4°C.

ii) *Preparation of sensitised SRBCs*

Defibrinated SRBCs are preserved by mixing in an equal volume of Alsever's solution. These can be stored at 4°C for up to 4 weeks. Wash 25 ml of the stock SRBCs with 25 ml of VBS. Centrifuge at 400 ***g*** for 10 minutes. Aspirate off the VBS and resuspend in 50 ml of VBS. Repeat the wash a total of three times. Following the final wash, dilute the SRBCs at a ratio of 2.2 ml of packed SRBCs to 98 ml of VBS. The SRBCs can then be standardised by optical density: mix 1 ml of the diluted, washed SRBCs with 14 ml distilled H_2O, determine the absorbance using a spectrophotometer, and standardise to 0.25 at a wave length of 550 mm. The reading obtained can be used in the following formula to determine the dilution needed:

$$\text{Final volume of SRBCs} = \frac{(\text{absorbance reading} \times \text{current volume})}{0.25\ \text{desired absorbance}}$$

The SRBCs are sensitised by rapidly adding an equal volume of VBS containing the appropriate dilution of haemolysin (dilution determined by titration). Incubate at 37°C for 15 minutes prior to use.

iii) *Veronal buffer saline*

VBS is prepared as a 5 × stock solution and diluted 1/5 with distilled H_2O prior to use. The following formula makes 4 litres. To distilled water add sodium barbital (7.5 g); barbital H_2O (dissolve in boiling H_2O) (11.5 g); $MgSO_4.7\ H_2O$ (4.056 g); NaCl (170.0 g); and $CaCl_2$ (0.078 g). Add distilled H_2O to make to 4 litres.

iv) *Complement titration*

Complement (C') is unstable and will deteriorate if improperly handled. Normally it should be kept frozen at –70°C in aliquots that are used at one time to eliminate refreezing. To obtain the desired working concentration (2 units per test well) first add 5% normal chicken serum for the modification to enhance sensitivity as described earlier. Then estimate a starting point based on previous lots. A good starting point is a dilution of 1/30 after the chicken serum has been added. Set up a series of tubes with various amounts of complement in VBS. The VBS should contain the antigen to be used in the reaction and take into account any anticomplementary properties of the antigen. A common method is to dilute 0.10 ml C' + 0.90 ml VBS; 0.12 ml complement + 0.88 ml VBS, etc. through 0.25 ml C' + 0.75 ml VBS. Incubate the tubes for 2 hours in a 37°C water bath. Add 0.5 ml of sensitised SRBCs to each tube. Incubate for 1 additional hour in the 37°C water bath. The highest dilution giving complete haemolysis equals 1 unit. Twice that amount equals 2 units. The following formula can be used to obtain 2 units/0.05 ml:

$$x = (di)\ (v)/2dh$$

where:

x = reciprocal of C' dilution desired to yield 2 units C'/well
di = reciprocal of C' initial dilution used in titration (1/30)
v = volume of diluted C' to be added
dh = twice the volume of C' giving complete haemolysis in titration

v) *Titration of haemolysin*

Haemolysin can be obtained from commercial sources. It must be standardised by titration. The following procedure is recommended:

Prepare a 1/100 dilution of the stock haemolysin in VBS. From this, prepare 1/300, 1/400, and 1/500 dilutions in tubes. From each of these dilutions, make 0.5 ml of twofold dilutions in VBS for a block titration.

To determine haemolysin concentration, add the following to 0.5 ml of each dilution: 0.5 ml of C' at 1/30 dilution, 0.5 ml of unsensitised SRBCs at 0.25 optical density, and 1.5 ml of VBS. Incubate for 1 hour at 37°C, and then centrifuge at 400 ***g*** for 5 minutes. One unit of the haemolysin is the dilution that gives complete lysis of the SRBCs. The haemolysin solution is prepared in VBS at the dilution containing 2 units of haemolysin. This is then added to an equal volume of SRBCs at the proper concentration.

vi) *Titration of antigen and positive control serum*

In order to standardise the CF test, it is also necessary to have titres of both the antigen and the positive control serum. If the titre is known for either the positive serum or antigen, the titre of the other component can be determined by performing the CF test using dilutions of the component being titred. If titres of both the positive serum and antigen are unknown, a block titration (chequerboard) can be used to determine the limiting dilutions of both the antigen and the antibody where haemolysis starts. It is very critical to obtain these titres accurately.

For both the antigen and the positive control serum, 4 units are used. A unit is the highest dilution that will give a positive test. That is, if a dilution of 1/160 gives a positive test, then a 1/40 dilution has 4 units and is used for the test.

Complement-fixing antibodies usually appear within 7–10 days of infection. For a positive diagnosis, a four-fold rise in CF antibody titre is required. A presumptive diagnosis by serological tests on a flock can only be made if typical clinical signs are present and a majority of the birds have antibody titres of >1/64.

b) Other tests

Other serological tests have been developed, but their specificity has not yet been sufficiently evaluated. The ELISA for group-specific chlamydial antibodies is more rapid and sensitive than the CF test; it can be automated. Evaluations of ELISAs for the detection of antibodies to both *C. trachomatis* and *C. psittaci* indicate that the tests are highly sensitive but lack specificity. New tests are being developed that use peptides or recombinant antigens which may correct the specificity problem (Sachse *et al.,* 2009b).

Other tests include the agar gel immunodiffusion test (Page, 1974), the latex agglutination (LA) test, the elementary body agglutination (EBA) test (Grimes & Arizmendi, 1996; Grimes *et al.,* 1994) and the micro-immunofluorescence test (MIFT). Immunodiffusion is less sensitive than the CF test. The LA test will detect antibodies to *C. psittaci*, and is easy and rapid to perform (Grimes *et al.,* 1993). Latex beads are coated with purified chlamydial antigen, mixed thoroughly with the test serum on a glass plate, and rotated for 2 minutes to enhance agglutination. The test is read against a dark background. Sera giving positive reactions should be retested with uncoated beads to eliminate possible nonspecific agglutination. The LA and direct CF tests correlate in 72.5% of tests with paired sera. The LA test has a sensitivity of 39.1% and a specificity of 98.8% relative to the direct CF test (Grimes *et al.,* 1993). The test detects both IgM and IgG, but it is best at detecting IgM. It has been suggested for use in detecting recent or active infections. The EBA test detects only IgM, and it is indicative of a current infection. The MIFT is rapid and easy to perform; however, fluorescence-conjugated anti-species sera are not always available.

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

There are no commercial vaccines available for chlamydiosis in poultry. Attempts to produce a vaccine have met with limited success, and most have been based on bacterins produced by formalin inactivation of concentrated suspensions of chlamydiae. There is evidence that immunity involves cell-mediated immune responses (Beeckman, & Vanrompay, 2010; Smith *et al.,* 2005), but vaccine manufacture has not been directed towards reactions of this type.

~~Antibiotics are the only current means of control. *Chlamydophila* *Chlamydia* *psittaci* is susceptible to a number of antibiotics: the drug of choice varies from country to country. Chlortetracycline, doxycycline, and other tetracyclines are the most commonly used. Treatment needs to be maintained for extended periods of time. For pet birds, 45 days is often recommended (Smith *et al.,* 2005; Vanrompay *et al.,* 1995).~~

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* *

NB: There is an OIE Reference Laboratory for avian chlamydiosis (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).

ADVICE FOR MEMBER COMMENTS

Chapter Number and Title: 2.3.11. Fowl typhoid and Pullorum disease

Country making the comments

Date

It would be appreciated if the following guidance notes are followed in making a reply:

1. *Comments may be general or specific, but specific comments are usually more valuable. General comments should be such that some conclusion and action can be taken in response to them. For example, it is of little help to make statements such as "This test is no longer used in our laboratory", without indicating the reasons and what test is used instead.*

2. *Specific comments should be identified, by indicating the line number in the text, to facilitate the making of editorial changes.*

3. *The pointing out of typing or technical errors is welcome, but the correct word or figure should be indicated. For example, it is of little help to say that 0.8 M is too high, if the preferred value is not indicated.*

4. *Bear in mind that the introductory chapters are not intended to be exhaustive, and indeed none of the chapters can give a completely comprehensive cover of the subject, otherwise the* Terrestrial Manual *would be too long. However, assistance in indicating priorities is always helpful.*

5. *The* Terrestrial Manual *is intended for world-wide use. The chapters need to reflect the development of new technology, but the old established methods, usually requiring less sophisticated apparatus, have to be retained. New technology should not be described in detail until it has gained wide acceptance as a reliable method.*

General Comments

Specific Comments (*add continuation sheets if required*)
line:

CHAPTER 2.3.11.

FOWL TYPHOID AND PULLORUM DISEASE

SUMMARY

Pullorum disease of chickens is a bacterial infection caused by Salmonella enterica *subspecies* enterica *serovar Gallinarum biovar Pullorum* (Salmonella *Pullorum*)[1]. *At this time the serovar is referred to as Gallinarum in some parts of the world and Pullorum in others; in this chapter the serovar will be referred to as Gallinarum or Pullorum according to the biovar under discussion. In its acute form,*

Pullorum disease is almost exclusively a septicaemic disease of young chickens. However, the organism may also be associated with disease in turkey poults and may be carried subclinically or lead to reduced egg production and hatchability plus a range of atypical signs in older birds. Ovarian transmission is a major route by which the organism can spread. Game birds and 'backyard' poultry flocks may act as reservoirs of infection, and wild birds may act as vectors for the organism and as such are important in the epidemiology of the disease.

Fowl typhoid in chickens and turkeys is caused by S. *Gallinarum biovar Gallinarum and is more often observed in the later growing period and in mature stock. Disease is often characterised by rapid spread with high morbidity and acute or subacute mortality. Red mites may be involved in the transmission of disease and persistence in poultry houses*

Clinical signs in chicks and poults include anorexia, diarrhoea, dehydration, weakness and death. In mature birds Pullorum disease is less severe but decreased egg production, poor hatchability and some increased mortality may occur. Fowl typhoid is a more acute septicaemic condition which mainly affects mature birds and may be particularly severe in commercial laying flocks.

Salmonellosis caused by Salmonella bongori *or ~~other~~ subspecies of* Salmonella enterica *is covered in chapter 2.9.9 of this* Terrestrial Manual.

Identification of the agent: *Samples should not be taken from birds or eggs that have recently been treated with antimicrobial drugs. Swabs or aseptically collected samples from infected tissues, or intestinal and cloacal contents should be used for diagnostic testing. Other materials that may be sampled include eggs, embryos, faecal droppings and hatcher debris, especially fluff, dust and broken eggshells and chick box linings. Samples of tissues such as caecal tonsils and spleen from infected birds are preferable to faecal and environmental samples. Tissue samples should be inoculated into non-selective and selective enrichment broths and on selective agar medium, such as brilliant green agar, as soon as possible after collection. In case of delay, samples should be stored at 4°C. Typical colonies can be identified by serological and biochemical tests.*

Serological tests: *These are satisfactory for ~~establishing~~ identifying the presence and estimating the prevalence of infection within a flock. The test used in the field is the rapid whole blood plate agglutination test. This test is unreliable in turkeys and ducks as many uninfected birds may give positive reactions. In the laboratory a serum agglutination test is used, either as a rapid plate test or as a tube test. These can be applied as macro- or microagglutination tests though the latter may be more likely to give false-positive results with turkey sera. Any positive reactors should be confirmed as being infected by culture at post-mortem examination. Enzyme-linked immunosorbent assays have been reported but no commercial test is available.*

The use of vaccines to control S. *Enteritidis or S. Gallinarum infections in chickens may cause problems in the interpretation of serological results.*

1 See the note in Chapter 2.9.9 Salmonellosis for the principles followed concerning the nomenclature of *Salmonella*.

Requirements for vaccines ~~and diagnostic biologicals~~: *Live and inactivated vaccines are available for fowl typhoid in some countries. The most commonly used vaccine is a commercial live vaccine derived from the stable rough strain of S. Gallinarum known as '9R'.*

A. INTRODUCTION

Fowl typhoid and pullorum disease, caused by *Salmonella enterica* subspecies *enterica* serovars Gallinarum biovars Gallinarum and Pullorum, respectively, are widely distributed throughout the world but they have been eradicated from commercial poultry in many developed countries of Western Europe, the United States of America (USA), Canada, Australia and Japan. In the United States and the United Kingdom the serovar is referred to as Pullorum (Hitchner, 2004), even though the strains are now considered to be the same serovar that is derived from *S.*Enteritidis by gene deletion events (Thomson *et al.*, 2008); in this chapter the terms serovar Gallinarum or Pullorum will be used. ~~However,~~ *S.* Gallinarum has recently recurred in some European countries (Ivanics *et al.,* 2008). *Salmonella* Pullorum remains as a constant reservoir in wild and game birds. *Salmonella Gallinarum* and *S.* Pullorum are host adapted to avian species (Eswarappa *et al.,* 2009) and are considered to pose a minimal zoonotic risk (Shivaprasad, 2000), although the genome is continually evolving, which could theoretically widen the host range in future (Liu *et al*., 2002). Non-typhoidal *Salmonella* serovars are normally handled in the laboratory at containment level (CL) 2, but *Salmonella* Typhi and Paratyphi are handled at CL3. In some laboratories, non-typhoidal serovars that are more likely to cause systemic disease and mortality in humans, e.g. *S.* Choleraesuis, may also be processed at CL3.

Salmonellosis caused by *Salmonella bongori* or ~~other~~ subspecies of *Salmonella enterica* is covered in chapter 2.9.9 of this *Terrestrial Manual*.

Clinical signs of fowl typhoid are typical of a septicaemic condition in poultry and include increased mortality and poor quality in chicks hatched from infected eggs. Older birds show signs of anaemia, depression, laboured breathing and diarrhoea causing adherence of faeces to the vent. The highest mortality occurs in birds of 2–3 weeks of age. In older birds disease may be mild or inapparent. In breeding and laying flocks susceptibility is increased at the point of lay (Wigley *et al.,* 2005), but reduced egg production and hatchability may be the only signs of *S.*Pullorum. Trans-ovarian infection resulting in infection of the egg and hatched chicks or poults is one of the most important transmission routes for the disease.

Post-mortem signs of pullorum disease in newly hatched chicks are those of peritonitis with generalised congestion of tissues and an inflamed unabsorbed yolk sac. Longer standing infections commonly lead to typhlitis with development of necrotic caecal casts and small necrotic foci in the liver, lungs and other viscera. Small lesions in the liver and spleen of Pullorum-infected birds may show a 'white spot' appearance that is not seen with Gallinarum; however, this lesion is not pathognomic. These *Salmonella* are very poor at colonisation and survival in the gastrointestinal tract is often indicative of later stages of clinical disease. Adult birds may develop misshapen or shrunken ovaries with follicles attached by pedunculated fibrous stalks. Variant strains of *S.* Pullorum do not normally cause clinical disease or may result in mild, nonspecific signs but may lead to seroconversion.

In fowl typhoid, as well as generalised signs of septicaemia, the liver is usually enlarged, dark and friable with a distinctive coppery bronze sheen that may only develop after exposure to air. The bone marrow is also often dark brown. Although clinical signs and post-mortem findings of pullorum disease and fowl typhoid may be highly suggestive of the conditions, they are not sufficiently distinct from other causes of septicaemia to be pathognomic. It is therefore necessary to confirm disease by isolation of the organisms. Serological tests can be used to establish the presence of the disease in a flock.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

o Bacteriological culture methods

In its acute form, pullorum disease is almost exclusively a disease of young chickens, and the agent can be recovered from almost all organs, tissues and faeces. In older birds that have become carriers, *S.* Pullorum is most commonly recovered from the ova and oviduct; and it is recovered only occasionally from other organs and tissues, including the alimentary tract. In the acute phase of fowl typhoid the organism is also widely distributed, but in carrier birds, the organism is found most often in the liver, spleen and reproductive tract, and occasionally in the caecal tonsils.

~~o Culture~~

Salmonella Pullorum and *S.* Gallinarum belong to the Kauffmann–White scheme serogroup D, along with *S.* Enteritidis, which is ~~thought to be~~ closely related (Grimont & Weill, 2007). The organisms are Gram negative non-sporogenic rods 1.0–2.5 µm in length and 0.3–1.5 µm in width. They are considered to be non-motile under normal conditions but inducement of flagellar proteins and motility has been shown in some strains of *S*. Pullorum when grown in special media (Holt & Chaubal, 1997).

For optimal recovery of the organisms, the birds being sampled should not have been treated with antimicrobial drugs for approximately 2–3 weeks previously.

Samples may be obtained from live birds, preferably after identifying them as highly sero-positive birds. Fresh or freshly chilled carcasses, egg materials, fresh faeces, or any contaminated materials from housing, incubators or transport boxes may also be taken, but faecal and environmental samples often fail to reveal the presence of the organism because of inconsistent shedding and the poor sensitivity of bacteriological detection methods. Swabs may be taken from the cloaca of sick live birds but post-mortem tissues are preferable. Samples from visibly abnormal tissues are ~~preferable to faecal and environmental samples~~ preferred, but aseptically gathered samples can also be taken from the spleen, liver, gall-bladder, kidneys, lungs, heart, ova, testes, alimentary tract or joint lesions. The preferred tissues for routine investigation are liver, ileo-caecal junction and ovaries/oviduct. The surface is seared with a hot spatula and a sample is obtained by inserting a sterile cotton swab or sterile loop through the heat-sterilised surface. The demonstration of infection in serological reactor birds that are apparently normal may, in some cases, require the culture of large volumes of homogenised tissues as well as direct swabbing. Tissue pools may be made from tissues collected from a number of birds.

When floor litter, ~~or~~ faecal material or the hatchery is sampled, it should be remembered that *S.* Pullorum and *S.* Gallinarum in low numbers associated with subclinically infected carrier birds are more difficult to isolate from faecal and environmental samples than other salmonellae and it is always preferable to culture sick or recently deceased birds~~. Environmental samples may include sock or boot swabs, floor faeces, moist and dry litter and swabs from open drinkers.~~ Red mites associated with poultry which are infected with *S*. Gallinarum often contain the organism after feeding and can be cultured. These samples should be cultured by direct inoculation of a selective enrichment broth such as selenite cysteine, followed by plating on selective media such as brilliant green agar (Parmar & Davies, 2007).

Both *S.* Pullorum and *S.* Gallinarum grow well in pure culture on non-selective media, but selective and enrichment media have been described that contain substances to inhibit the growth of extraneous organisms. *Salmonella* Pullorum may grow slowly and produce very small colonies on selective media so incubation of plates for 48 hours is recommended. The efficiency of recovery of *Salmonella* varies according to circumstances, and experience in the use of a medium is an important but unquantifiable factor. Some complex media may have an inhibitory effect on these organisms, so that it is advisable to use both non-selective and selective media for isolation from tissues. Both solid media and broths can be employed. As the toxic properties of selective media may vary, it is preferable to monitor these by comparing growth of control cultures on both types of medium. The inhibitory media should grow at least 75% of the colonies of the corresponding non-inhibitory medium (Ellis *et al.*, 1976; Mallinsen & Snoeyenbos, 1989)

All the media mentioned below are examples of commonly used media, but there are many others found to be equally satisfactory.

Non-inhibitory media include nutrient agar and blood agar, on which colonies are seen to be smooth, translucent, slightly raised, and about 1–2 mm in diameter. *Salmonella* Gallinarum grows more rapidly than *S.* Pullorum and produces larger colonies with a distinctive smell resembling that of seminal fluid on most media. Broths include buffered peptone water and nutrient and meat infusion broths or universal pre-enrichment broth.

o Selective media ~~include~~:

MacConkey agar: the agar is inhibitory to non-enteric organisms, it differentiates lactose fermenters (pink colonies) from non-lactose fermenters (colourless colonies). NaCl is omitted to limit the spread of *Proteus* colonies. *Salmonella* colonies are smooth and colourless. *Salmonella* Pullorum produces smaller colonies than other salmonellae. MacConkey is the agar of choice for direct plating from tissues.

Xylose lysine deoxycholate agar: the agar is inhibitory to non-enteric organisms. *Salmonella* Pullorum grows sparsely as small red translucent colonies. *S.* Gallinarum colonies are small, dome-shaped, and may have a central black spot due to H_2S production, but this reaction may be delayed or variable.

Brilliant green agar (*BGA*): the agar is inhibitory to coliforms and most *Proteus* strains; useful for distinguishing enteric organism colonies. Salmonellae form low, convex, pale red, translucent colonies of 1–3 mm in diameter, similar to *Citrobacter*. *Proteus* forms pin-point colonies, *Pseudomonas aeruginosa* appears

as small red colonies, and lactose fermenters are green. *Salmonella* Pullorum produces smaller more pale colonies than other salmonellae. BGA is the agar of choice following enrichment.

Brilliant green sulphapyridine agar: the agar is inhibitory to coliforms and *Proteus* strains. The sulphapyridine is added to stabilise selectivity in the presence of egg materials. *Salmonella* Pullorum produces small colonies.

Salmonella Pullorum and Gallinarum grow poorly and do not produce typical colonies on newer chromogenic agars such as Rambach agar.

- **Liquid enrichment and selective media ~~include~~:**

Selenite F broth: inhibitory to coliforms but not *Proteus*, improved by addition of brilliant green. Loss of activity after 24 hours. Selenite cysteine broth is more stable. Although selenite broths are considered to be preferable for isolation of *S.* Pullorum and *S.* Gallinarum from faeces, if there are difficulties with issues of toxicity or shelf life in particular laboratories the other enrichment broths mentioned below may be used. Most of these other broths are however designed to be used following a non-selective enrichment stage and *S*. Gallinarum and *S*. Pullorum are readily overgrown by competitor organisms in non-selective faecal culture resulting in false-negative tests. Direct selective enrichment is therefore recommended for faeces and intestinal or environmental samples. Non-selective enrichment may give better results for tissues obtained by aseptic post-mortem where there should be no competing organisms (Mallinson & Snoeyenbos, 1989).

Tetrathionate/brilliant green broth: inhibitory to coliforms and *Proteus*, but may also inhibit some strains of *S.* Pullorum/Gallinarum.

Rappaport–Vassiliadis soya (*RVS*) *peptone broth:* for selective enrichment following pre-enrichment, use 1 part inoculum to 100 parts medium. *Salmonella* Pullorum and Gallinarum are more likely to be overgrown by other organisms during pre-enrichment of faeces or intestinal contents than salmonellae that are not host-adapted so direct enrichment with RVS may also be attempted.

- **Recovery of salmonellae**

The methods for recovering *S.* Pullorum and *S.* Gallinarum vary according to the origin of the samples. Although their isolation from cloacal swabs and faeces may be unrewarding, examination of tissues taken at post-mortem is usually more successful. The methods are as follows:

Cloacal swabs and fresh faeces from live birds: swabs dipped in nutrient broth are suitable, small swabs being used for young chickens. The swabs are streaked on selective media, and placed in enrichment broth. The plates and the broth are incubated at 37°C. At this temperature, *Proteus* and *Pseudomonas* organisms tend to be inhibited relative to *Salmonella*. Higher temperatures may be used with some broths, e.g. 41.5°C for Rappaport–Vassiliadis (RV). Subcultures are made on to selective media after 24 and 48 hours.

Gall-bladder contents: swabs of gall-bladder contents are streaked on to non-selective and selective agars and placed in inhibitory and non-inhibitory broths, followed by incubation at 37°C and subculture on to selective agar after 24 and 48 hours.

Organs and tissues: swabs or segments of tissues are taken in an aseptic manner from individual tissues and lesions and cultured on to non-selective and selective media, and into similar non-selective and selective broths. These are incubated at 37°C and subcultured on to selective agar after 24 and 48 hours. Intestinal material in selective broths may also be incubated at 40°C; *S.* Gallinarum grows well but there may be some inhibition of *S.* Pullorum at this temperature.

Carrier birds: larger amounts of material may be required to identify the carrier birds. The ovary and oviduct are the tissues of choice for *S.* Pullorum, and the liver, ~~and~~ gall-bladder and caecal tonsils as well as ovary and oviduct should be tested for *S.* Gallinarum. In practice it is usually best to pool samples from a variety of tissues including the spleen, but intestinal tissues should not be pooled with other tissues. Tissues are homogenised in a small volume of broth and directly plated out. Approximately 10 ml of homogenate is also added to 100 ml of non-selective enrichment broth (e.g. buffered peptone water) and selective enrichment broth (e.g. selenite cysteine broth or brilliant green broth), and incubated at 37°C. These broths are subcultured on to non-selective and selective agar after 24 hours.

Alimentary canal, including the caecal tonsils and intestinal contents: after grinding or homogenisation in a small volume of broth, 10 ml of the homogenate is incubated in 100 ml of selective enrichment broth at 37°C. In general better isolation is achieved with selenite cysteine broth.

Eggshells: the broken eggshells are placed in a tenfold volume of enrichment broth (e.g. selenite cysteine broth). The broth is incubated at 37°C and subcultured on to selective agar after 24 and 48 hours.

Egg contents: Aseptically taken contents of fresh eggs are homogenised and mixed with 200 ml of buffered peptone water or nutrient broth, incubated at 37°C, and subcultured on to non-selective and selective agar after 24 and 48 hours. Incubated eggs, whether infertile or containing small embryos, can be similarly treated.

Embryos: Homogenised viscera and swabs from the yolk sacs of well developed embryos may be streaked on to non-selective and selective agar, one swab being placed in 10 ml of both non-selective and enrichment broth (e.g. selenite cysteine broth or brilliant green broth). Incubation is carried out at 37°C, and subcultures are made on to non-selective and selective agars after 24 and 48 hours.

Environmental samples: These include hatcher fluff, debris and macerated egg/chick waste samples and chick box liners or floor faecal or litter samples; 25 g is mixed with 225 ml of enrichment broth (e.g. selenite cysteine broth, brilliant green broth), incubated at 37°C, and subcultured on to selective agar after 24 and 48 hours.

Polymerase chain reaction (PCR) based tests may also be used, but have not been fully validated internationally (Cha *et al.,* 2008; Oliviera *et al.,* 2003).

~~1. Identification of the agent~~

o Confirmatory procedures

Typical *S.* Gallinarum colonies on non-selective media are round, translucent, glistening, domed, smooth, and 1–2 mm in diameter after 24–48 hours' incubation. *Salmonella* Pullorum colonies are slightly smaller and translucent. On selective media their appearance varies with the medium, but suspect colonies can be investigated serologically by testing for 'O'9 somatic antigens, observing for motility and testing biochemically.

After incubation for 20–24 hours, the plates should be examined carefully for the presence of typical *S.* Pullorum and *S.* Gallinarum colonies. If growth is slight after 24 hours' incubation, the plates should be re-incubated for a further 24 hours and examined again. For biochemical and serological confirmation from each plate, five typical or suspect colonies should be chosen for further examination. If there are fewer than five typical or suspect colonies, all of them should be taken for further examination. Selected colonies should be streaked on to the surface of nutrient agar, in a manner that allows the growth of separate colonies. For biochemical confirmation, only pure cultures taken from non-selective media should be used. The following media should be streaked using an inoculating loop: triple sugar iron (TSI) agar; lysine iron agar (or l-lysine decarboxylation medium); urea agar according to Christensen; tryptone/tryptophan medium for indole reaction; glucose with an inverted Durham tube for acid and gas production; dulcitol, maltose, ornithine decarboxylation medium and semisolid agar, for motility. The reactions shown in Table 1 occur.

Identification kits are commercially available, for example Analytical Profile Index (API) system for Enterobacteriaceae. However, care must be taken when using API because *S.* Pullorum may be misidentified as *Hafnia* spp. Molecular tests using ribotyping techniques and PCR have been developed in research laboratories (Kang *et al.,* 2011), but are not generally available for confirmation of *S.* Gallinarum and *S.* Pullorum.

For serological confirmation to serogroup level, colonies from non-selective media (nutrient or blood agar) are used. The first stage is elimination of autoagglutinable strains. For this, material taken from a single colony of pure culture is transferred to a glass slide and mixed with a drop of sterile saline. The slide is rocked gently or the drop stirred with a loop for 30–60 seconds and observed for agglutination against a dark background, preferably with the aid of a magnifying glass or dissecting microscope. If the bacteria have clumped into more or less distinctive units, the strain is considered to be autoagglutinable and must not be submitted to the following tests. If the bacterial sample is recognised as non-autoagglutinable, it is tested with a polyvalent 'O' (A–G) antiserum. For this purpose, the material from a single colony is dispersed in the drop of polyvalent 'O' antiserum on the glass slide to obtain a homogenous and turbid suspension. After gently rocking for 30–60 seconds, the reaction is observed against a dark background for agglutination. Alternatively the slide agglutination test may be carried out with smaller volumes of suspension under a dissecting microscope. In this case a portion of the colony to be checked is added to a loopful of saline on the microscope slide to produce a light suspension to check for autoagglutination ('rough strains'). If no agglutination takes place, one or two loops of antisera are added, the drop stirred with a loop and observed for agglutination. *Salmonella* Pullorum and *S.* Gallinarum should agglutinate with polyvalent 'O' antisera but not with polyvalent flagella (poly 'H' phase 1 and phase 2) antisera. If the reaction is positive, the single colony is tested further in the same manner using group-specific sera for *S.* Pullorum and *S.* Gallinarum serotypes ('O'9 antiserum). After serogrouping, isolates may be sent to a reference laboratory for serotyping.

Table 1*. Biochemical investigation of* Salmonella *Pullorum and S. Gallinarum*

	Salmonella Pullorum	*Salmonella* Gallinarum

TSI glucose (acid formation)	+	+
TSI glucose (gas formation)	v	–
TSI lactose	–	–
TSI saccharose	–	–
TSI hydrogen sulphide	v	v
Gas from glucose (medium with Durham tube)	+	–
Urea hydrolysis	–	–
Lysine decarboxylation	+	+
Ornithine decarboxylation	+	–
Maltose fermentation	– or late +	+
Dulcitol	–	+
Motility	–	–

+ = 90% or more positive reaction within 1 or 2 days; – = No reaction (90% or more); v = Variable reactions.

It is also possible to confirm and differentiate *S.*Gallinarum by specific PCR (Shah *et al.,* 2005).

o Test procedure for culture of visceral, faecal, intestinal and environmental samples for *S.* Pullorum and *S.* Gallinarum

i) Where possible, begin laboratory procedures on the same day as samples are collected.

ii) Homogenise the material as much as possible by manual mixing, gentle macerating or stomaching with a small volume of sterile saline if the material is dry.

iii) Stir the mixture with a small rectal swab or loop and streak thickly on to one-quarter of a brilliant green agar plate. (Swabs from uncontaminated tissues sampled in an aseptic manner can also be streaked on to blood agar.)

iv) From this deposit of material on the plate, streak the rest of the plate to obtain individual colonies.

v) Add 5–25 g of the homogenised sample to freshly made selenite cysteine broth (see note on liquid enrichment and selective media above) to make a 1:10 sample to broth ratio. Shake or stir to disperse the sample in the broth.

vi) Incubate the brilliant green agar plates and selenite cysteine broth at 37°C for 24 hours.

vii) Examine the plate after 24 hours' culture. Carry out agglutination tests on up to five suspect colonies with polyvalent 'O' (A–G) antisera and polyvalent H (phase 1 and phase 2) antisera. If agglutination is unclear subculture suspect colonies on to nutrient agar or blood agar and repeat tests after 24 hours' incubation of those media.

viii) If poly 'O' is positive then check with 'O'9 antiserum. If 'O'9 is positive and poly 'H' is negative, this is indicative of the possible presence of *S.* Pullorum or *S.* Gallinarum.

ix) If there are no positive colonies on the brilliant green agar plate, streak out a 10 µl loop of incubated selenite cysteine broth onto brilliant green agar as in step iv above.

xi) Incubate the brilliant green agar plates at 37°C for 24 hours and re-incubate the previous (negative) brilliant green agar plates and the selenite cysteine broths for a further 24 hours.

xi) Repeat examination of plates as in step vii above.

xii) If plates are still negative, re-plate from selenite cysteine broth and incubate brilliant green agar plate, that was inoculated in step ix, for a further 24 hours and examine as in step vii above.

xiii) Confirm *S.* Pullorum and *S.* Gallinarum using biochemical tests as shown in Table 1. Isolates can be sent to a *Salmonella* reference laboratory for confirmation of serotype and for phage typing of *S.* Pullorum.

o Molecular epidemiology

Standard molecular 'fingerprinting' techniques used for *Salmonella*, such as plasmid profile analysis, pulsed field gel electrophoresis or ribotyping can be used for investigating outbreaks of *S.* Pullorum or *S.* Gallinarum. It is often necessary to use combinations of such methods to obtain maximum discrimination. High throughput sequencing has also been applied to *S.* Gallinarum.

2. Serological tests

Serological tests are best applied as a flock test as results for individual birds will vary according to the stage of infection. It is therefore necessary to take sufficient individual samples to determine infection in the flock. The number of samples will depend on the expected prevalence and level of confidence desired (see Chapter 1.1.1 Collection and shipment of diagnostic specimens). If the test is to be used for detecting individual infected birds for culling, it should be repeated at least twice and preferably until the whole flock has given at least two negative tests.

The tests that are most readily applied include rapid whole blood agglutination, rapid serum agglutination (RST), tube agglutination and micro-agglutination (USDA, 1996). Other invasive *Salmonella* such as *S.* Enteritidis and *S.* Typhimurium or use of vaccination may lead to false-positive results in serological tests for *S.* Pullorum.

Both *S.* Pullorum and *S.* Gallinarum possess 'O' antigens 9 and 12 and may also possess O antigen 1 (Brooks *et al.,* 2008). However, in the case of *S.* Pullorum, there is a variation in the ratio of 12_1, 12_2 and 12_3; the standard strain contains more 12_3 than 12_2, while the reverse is true of the variant form. Intermediate forms also exist. (There appears to be no such form variation in the case of *S.* Gallinarum.) As this variation occurs, it is necessary to use a polyvalent antigen in immunodiagnostic tests. The same antigen is used to detect both *S.* Pullorum and *S.* Gallinarum, but detection of the latter may be relatively poor (Proux *et al.,* 2002).

a) Rapid whole blood agglutination test

The rapid whole blood agglutination test can be used under field conditions for detecting both *S.* Pullorum and *S.* Gallinarum, and the reactors can be identified immediately. However, it is not reliable in turkeys as the test results in a significant proportion of false-positive results. Sera can be screened by rapid slide agglutination test and positive reactions confirmed by the more specific tube agglutination test. Chickens can be tested at any age, although some authorities specify a minimum age of 4 months (USDA,1996; Wray & Wray 2000) and positive results from chicks less than 4 weeks of age may be due to maternal antibodies.

o Preparation of stained antigen for the rapid whole blood or rapid serum agglutination test

Incubate one standard form strain of *S.* Pullorum (antigenic structure 9, 12_1, 12_3) and one variant form (antigenic structure 9, 12_1, 12_2) at 37°C and harvest separately until final mixing for the complete antigen.

Sow strains on to separate agar slopes, incubate at 37°C for 24 hours, emulsify growth with sterile normal saline and spread an inoculum over an agar plate to produce easily selected discrete colonies. For this the plates are incubated for 48 hours, a number of colonies are marked out and each is tested for agglutination on a slide with 1/500 acriflavine in saline. Smooth-phase colonies do not produce agglutination. Pick off typical colonies that do not produce any agglutination, seed on to agar slopes, and incubate for 24 hours. Emulsify the growth in saline and evenly distribute 2 ml over the surface of the medium (200 ml) in a Roux or similar flask. Incubate the flasks for 60 hours.

For harvesting the bacterial growth, flood the surface of each flask with enough sterile buffered formol saline, pH 6.5 (8.5 g/litre sodium chloride, 10 ml/litre neutral formalin, 4 ml/litre 0.5 M sodium phosphate: made up to 1 litre with distilled water, pH adjusted to 6.5 using 1 M orthophosphoric acid or 1 M sodium hydroxide), to give dense cell suspensions (about 10 ml per flask). Add 12–15 sterile glass beads of 3–5 mm diameter and rock the flasks until all the growth is in even suspension; leave in a vertical position for at least 15 minutes. Check the morphology and purity of the suspensions by preparing and examining Gram-stained films. Bulk the suspension from each flask containing the same strains. To each 100 ml of suspension, add 200 ml of absolute alcohol. Shake the mixture and allow to stand for 36 hours or until precipitation is complete. Check the agglutinability of the standard and variant precipitate by first centrifuging a sample to separate the alcohol, which is removed, dilute with normal saline and test with a known positive and negative serum. If satisfactory, remove the clear supernatant alcohol (centrifugation at 2000 *g* for 10 minutes may be helpful in precipitation), and add sufficient phosphate buffered saline (PBS) containing 10% (v/v) glycerol to standardise the density to 75 × No. 1 Wellcome opacity tube (or 50 × tube No. 1.0 on the McFarland scale). Add equal volumes of standard and variant strains, and add 1% (v/v) of 3% (w/v) alcoholic crystal violet solution to the final mixture, and allow to stand for 48 hours at room temperature. Store in a tightly closed container at 0–4°C for up to 6 months. To assess safety, carry out a culture test on blood agar for non-viability of the unwashed antigen before standardisation. Each bottle of antigen must be tested after alcoholic precipitation and before standardisation against standard titre antisera for *S.* Pullorum and *S.* Gallinarum, and against a negative serum. If possible, also test with known positive and negative serum and blood from positive and negative chickens.

Stained antigen products for the whole blood plate agglutination test are available commercially, and although there seems to be some slight differences in their sensitivity (Gast, 1997), it is unlikely that poultry flocks infected with the different variants of *S.* Pullorum would be missed.

o Test procedure

i) Use a clean white tile marked into squares of about 3 × 3 cm. If a tile with 3 × 4 squares is used, up to 12 blood samples can be tested at the same time.

ii) Place 1 drop (about 0.02 ml) of crystal-violet-stained antigen in the centre of each square.

iii) Obtain a sample of fresh whole blood. This is conveniently done by making a stab of a wing vein using a needle with a triangular point.

iv) Place an equal size drop of fresh whole blood next to a drop of antigen.

v) Mix the drops of antigen and blood using a fine glass rod, which is wiped clean between samples.

vi) Use a gentle rocking motion to keep the drops agitated for up to 2 minutes. Several tests may be carried out simultaneously on the same tile, but the drops should not be allowed to dry out during this time. In very warm conditions, larger drops may be required to avoid drying out.

vii) A positive reaction is indicated by easily visible clumping of the antigen within 2 minutes.

viii) A negative reaction is indicated by absence of clumping of the antigen within 2 minutes.

ix) Include known positive and negative control sera on each testing occasion, using them in the same way as the blood.

x) On completion of a set of tests, the tile is washed and dried, ready for further use.

In the absence of positive reactions, any doubtful reactions can only be interpreted in the light of the previous *Salmonella* testing history of the flock. Where there are positive reactors, any doubtful reactor should be regarded as positive. Also, recently infected birds may not show a typical positive reaction until they are retested after 3–4 weeks.

b) Rapid serum agglutination test

The RST is performed in the same manner, except that serum is substituted for whole blood. For export test purposes an initial screening of sera by RST followed by confirmation of positives by the tube agglutination test is the optimal approach. Ideally serum samples tested by any method should be tested within 72 hours of collection as nonspecific reactions may increase in older samples. Fresh samples can be frozen for later testing if a delay is unavoidable.

c) Tube agglutination test

Fresh serum from chickens, turkeys or other birds is used at an initial dilution of 1/25, obtained by mixing 0.04 ml of serum with 1.0 ml of antigen[2]. Positive and negative control sera are included in each test. The antigen is prepared from unstained *S.* Pullorum or *S.* Gallinarum cultures diluted to a concentration of No. 1 on the McFarland scale (as described above). The mixture is incubated at 37 or 50°C for 18–24 hours before reading. A positive reaction consists of a granular white deposit with a clear supernatant fluid; a negative reaction shows uniform turbidity. Samples positive at a dilution of 1/25 are retested at a higher range of dilutions and a titre of 1/50 is usually considered to be positive, although this figure seems to vary in the literature. In many cases a single dilution of 1/50 is used but this may fail to detect some flock infections if only small numbers of samples are taken.

d) Micro-agglutination test

This resembles the tube agglutination test, but requires much smaller volumes of reagents. The test is performed in microtest plates. Sera are first diluted by adding 10 µl of serum to 90 µl of normal saline, and then adding 100 µl of previously standardised stained microtest antigen to give a final dilution of 1/20. By titrating the serum in doubling dilutions and adding an equal volume of standardised stained antigen, an end-point (titre) can be obtained. The plates are sealed and incubated at 37°C for 18–24 or 48 hours. A positive reaction consists of a fine diffuse precipitation, whereas a negative reaction shows a button-like precipitate. Titres of 1/40 are usually considered to be positive but this test is more liable to produce false-positive results with turkey sera.

Other serological tests include micro-antiglobulin (Coombs), immunodiffusion, haemagglutination and enzyme-linked immunosorbent assay (ELISA).

ELISA techniques have been described for detecting antibodies to *S.* Pullorum and *S.* Gallinarum (Oliviera *et al.,* 2004). The indirect ELISA using lipopolysaccharide antigen is likely to be the most sensitive and specific serological flock test for *Salmonella*, including *S.* Gallinarum and *S.* Pullorum. It is relatively easy to perform with serum or yolk, and can be used for quantifying the titre of antibody (Barrow, 1992; 1994; Wray & Wray, 2000). No commercial ELISA kits for *S.* Pullorum and *S.* Gallinarum are currently available.

2 For preparation of small volumes of somatic antigens see Chapter 2.9.9 Salmonellosis.

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

1. Background

a) Rationale and intended use of the product

Although both live and inactivated vaccines have been prepared for use against *S.* Gallinarum (Paiva *et al.*, 2009), the vaccine most widely used is made from the rough 9R strain (Harbourne *et al.*, 1963). It is normally ~~has~~ only employed in chickens. The number of viable organisms per dose is important; these organisms can survive in vaccinated birds for many months and may be transmitted through the egg (and perhaps from bird to bird). Vaccination may reduce flock losses, but will not prevent infection with field strains. In addition, vaccination with 9R may sometimes precipitate high mortality in infected birds (Silva *et al.*, 1981), and may stimulate the production of transient antibodies. It is usual to vaccinate at 8 weeks and again at 16 weeks of age. Antimicrobials should be avoided before and after vaccination.

Currently available vaccines, however, have only a minor role to play in the control of fowl typhoid as they offer short-lived protection against clinical disease and limited or variable protection against infection. Autogenous or locally produced vaccines can also be used to control clinical disease, but care must be taken to avoid strain instability leading to reversion to virulence (Okamoto *et al.*, 2010). Control can best be achieved by biosecurity, hygiene, good management, monitoring and removal of infected flocks. Commercially available 9R vaccines are commonly used for reduction of *S.* Enteritidis in laying flocks (Lee *et al.*, 2005).

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements. Most vaccines are produced in highly industrial commercial processes and are regulated by national veterinary medicines licensing authorities. Smaller quantities of emergency herd vaccines or autogenous vaccines are produced by private laboratories, but each production has to be specifically licensed.

2. Outline of production and minimum requirements for conventional vaccines

~~1. Seed management~~

a) Characteristics of the seed

i) Biological characteristics

For killed or live vaccines, the bacterial strain should be an organism as closely related to currently circulating field strains as possible. It should be carefully chosen from cases of severe clinical disease, and virulence and antigen production should be assessed. It is best to evaluate a panel of potential strains in this way before testing the final selection. The final vaccinal strain should be identified by historical records and characterised by stable phenotypic and/or genetic markers. Living vaccinal strains should be marked by stable characters allowing distinction from wild strains. Markers such as resistance to antimicrobials, for example rifampicin, or auxotrophism may be used. Attenuation of virulence should be stable and preferably obtained by two independent defined mutations. The stability of live vaccine strains can be verified by regular checks using sensitive molecular fingerprinting and micro-array techniques.

Live fowl typhoid vaccine is a suspension of suitably attenuated living organisms of a rough strain of *S.* Gallinarum, e.g. 9R. The organisms in the vaccine give the biochemical reactions characteristic of *S.* Gallinarum. Colonies of a 24-hour culture prepared from the vaccine on nutrient agar plates are rough when examined by the acriflavine slide test. The culture should not contain the somatic antigens characteristic of the smooth forms of *S.* Gallinarum

ii) Quality criteria (sterility, purity, freedom from extraneous agents)

Sterility and purity

The vaccine strain must be checked as follows:

- Staining of a smear of bacterial suspension on a glass slide using Gram stain.
- Homogeneity of culture on non-selective media.
- Metabolic requirements as indicated by biochemical tests.
- Detection of markers, and phage type.
- Agglutination with specific antiserum.

- The vaccine culture and any adjuvants, preservatives or other materials must be microbiologically sterile and non-toxic at the concentrations used.

Safety

The LD_{50} (50% lethal dose) or ID_{50} (50% infectious dose) may be determined in chickens or, preferably, signs of more mild adverse reactions should be checked in the target species. Ten times the field dose of live vaccine or twice the dose for killed vaccines must be given to the target species at the recommended age and by the recommended route. The animals are observed for absence of adverse reactions. Stability and non-reversion to virulence after serial passages in susceptible species should be shown for live vaccines. It is also necessary to consider repeat vaccination. Live vaccine should be shown not to persist for long in vaccinated animals or be transmitted to milk or eggs that may be consumed, and the method of application should not present a hazard to operators. In the case of *S.* Gallinarum vaccine at least six healthy, susceptible (preferably specific pathogen free [SPF]) chickens, 8–16 weeks of age, are each injected subcutaneously with ten doses of vaccine, and are observed for at least 7 days; no local or systemic reaction should develop.

Efficacy

Laboratory experiments and field trials should be used to show that the vaccine is effective. The laboratory experiments consist of vaccination–challenge tests in the target species at the recommended dose and age. The efficacy data can also be used as the basis for a batch potency test. Field trials are more difficult to undertake with respect to testing efficacy because of difficulties with standardising the challenge and providing appropriate controls. In the case of *S.* Gallinrum 9R vaccine or similar vaccines at least fifteen healthy chickens, 8–16 weeks of age, of a brown layer hybrid breed, and taken from a stock that is free from *S.* Pullorum infection, are each injected subcutaneously with a quantity of vaccine corresponding to one field dose, i.e. 5×10^7 viable organisms. After an interval of 21–28 days, the vaccinated chickens and an equal number of similar unvaccinated chickens are deprived of food for approximately 18 hours. The chickens are then challenged by oral administration of 1 ml of a broth suspension containing 5×10^7 organisms of a virulent strain of *S.* Gallinarum mixed with 300 mg of a powder consisting of chalk (40%), light kaolin (43%) and magnesium trisilicate (17%). All the chickens are observed for 14–21 days. The vaccine passes the test if at the end of this period the number of surviving vaccinated chickens that show no macroscopic lesions of fowl typhoid at post-mortem exceeds by eight or more the number of similarly defined control chickens.

Environmental aspects

Live vaccine strains should be tested for their ability to persist in the environment and infect non-target species such as rodents and wild birds that are likely to be exposed. Prolonged survival of some live vaccines in faeces and litter may present an unacceptable environmental hazard when the material is removed from the animal houses. Live vaccines should not be used in commercial laying flocks during lay.

b) Method of manufacture

i) Procedure

The seed culture is propagated and maintained using suitable media, of which many have been described (in textbooks) for growth of Salmonella. The media used must not contain serum or animal tissues. Culture may be on solid medium, in Roux flasks, or in liquid medium, in which case large-scale fermentation equipment may be used. Iron limitation or low temperature incubation on a minimal media may enhance lipopolysaccharide (LPS) antigen production by the vaccine strain. In the case of *S.* Gallinarum (9R), the vaccine may be prepared by inoculation of a suitable medium, such as peptone broth, with a fresh culture of *S.* Gallinarum (9R) and incubation at 37°C for 24 hours, with agitation. The organisms are harvested by sedimentation or centrifugation.

Alternatively the organisms may be grown on and harvested from a solid medium, such as nutrient agar. In either case, the suspension is diluted in PBS solution, pH 7.0, and may be freeze-dried. The dose used per bird is between 5×10^6 and 5×10^7 organisms.

Vaccine must be produced in suitable clean rooms to which only approved personnel have access. Care must be taken to avoid cross-contamination between areas where live organisms are processed and other areas. Contamination from operators and/or the environment must be avoided and vaccine preparation should take place in a separate area from diagnostic culture work. Operators must not work with vaccine whilst ill and must not be subject to immunosuppressive conditions or medications. Personnel must be provided with protective clothing in production areas and in animal rooms.

Seed-lot cultures are prepared from the primary seed-lot, and the number of passages is dependent on the validation of the process. The vaccine may be prepared by inoculation of a suitable medium, such as nutrient broth, with a fresh culture and incubation on a shaker at 37°C for 24 hours, with or without aeration. The organisms are harvested by sedimentation or centrifugation. Alternatively, the organisms

may be grown on and harvested from a solid medium, such as nutrient agar. In the case of live vaccines, the suspension is diluted in PBS, pH 7.0, and may be freeze-dried.

The time of inactivation of dead vaccines should be at least 33% more than that taken to reduce the viable number to an undetectable level. The inactivation process must be applied to the whole volume of the vaccine cell harvest.

Preservatives, excipient for lyophilisation, stabiliser for multi-dose containers or other substances added to or combined with a vaccinal preparation must have no deleterious effect on the immunising potency of the product.

ii) Requirements for substrates and media

All chemicals and growth media used should be guaranteed to be fit for purpose and checked by the use of suitable controls.

iii) In-process controls

The following points require attention:

- Visual control of the suspension, homogeneity by Gram stain, culture on non-selective medium.
- Slide agglutination with specific antisera.
- Titration of bacteria by turbidimetry and/or plate count.
- Test of effective inactivation (killed vaccine) by plating on non-selective medium or use of a medium that gives optimum chance of recovery, e.g. production medium with neutralisation of the inactivating compound.
- Titration of viable bacteria (living vaccine) before and after lyophilisation.

iv) Final product batch tests

Sterility/purity

Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.9.

Safety

A laboratory test that has previously shown a correlation with safety in the target species may be used to determine the absence of deleterious effects on vaccinated animals. Each batch should be tested in the target species at the recommended age and route, using at least twice the field dose for killed vaccines and ten times the dose for live vaccines. Observations are made on any adverse effects on the demeanour and health of the vaccinated animals and an assessment may be made of tissue reactions at the injection site.

Batch potency

Potency is tested using vaccination–challenge assay in chickens and/or other species, including (if practicable) any other target species and immunological response in target species.

c) Requirements for authorisation

i) Safety requirements

Certain killed vaccines may occasionally cause reactions in vaccinated animals because of their LPS content or the adjuvant used, and likewise live vaccines should be used with caution in animals that are not completely health at the time of vaccination. It is often necessary, however, to vaccinate flocks in the face of clinical fowl typhoid. Vaccines may also cause swelling at the site of injection, particularly if an oil-emulsion adjuvant is used.

Target and non-target animal safety

Killed vaccines are assessed in a double-dose test, and live vaccines are assessed in a test using ten times the dose, ideally in the target species. Live vaccines should be proven to be harmless in relevant non-target species that could be exposed to vaccine excreted by vaccinated animals. As *S.* Gallinarum and *S.* Pullorum are host specific, non-target species are less of a concern.

Reversion to virulence for attenuated/live vaccines

Live vaccines shall be shown in replication tests in target species to not revert to virulent strains during a suitably large number of replications. Mutations, especially undefined mutations, should be shown to be stable, and checks on stability can be made by molecular fingerprinting methods or sequencing.

Although the risk is small it is wise not to use live vaccines in a country where the organism in the vaccine has been eradicated. Special care should also be taken to ensure that attenuated vaccines are not incompletely attenuated or contaminated with seed organisms.

Environmental consideration

Live vaccines should not be able to replicate in the environment or persist for more than a short period.

ii) Efficacy requirements

For animal production

The duration of immunity is likely to vary considerably between products, vaccination regimes and individual vaccinated animals. The vaccine should provide protection throughout the laying period, and this can be measured by potency (efficacy) tests at stages during lay. A booster dose during lay may be required, but should not be used during lay in flocks providing eggs for human consumption.

Immunity to *Salmonella* is normally serovar or serogroup specific. Consultation among colleagues suggests that most killed vaccines will provide some protection for 6 months, while some live vaccines given by injection may elicit stronger immunity, which may persist for 1 year or more. It should be remembered, however, that a strong challenge such as that associated with continuously occupied farms or infected wild birds and mite populations may overwhelm vaccinal immunity and commercial live vaccines may be attenuated to reduce environmental survival in a way that reduces the immune response. There may also be problems with ensuring effective oral administration with live vaccines that are delivered orally or accuracy of injection with killed and live injectable vaccines. The Salmonella vaccines are intended to limit the extent of clinical disease in poultry, and also to reduce the risk of introduction of infection to flocks. If possible, the potency test should relate to the efficacy of the vaccine in the target species, and suitable criteria should be applied for passing batches. It may be possible to assess killed and injected vaccines by the antibody response produced, although it should be remembered that serum antibodies are only part of the host's protective mechanism against Salmonella. Alternatively, the potency of the vaccine may be assessed by its effect on challenged vaccinated animals compared quantitatively and statistically with unvaccinated controls.

For control and eradication

Vaccines for Salmonella are not capable of eradicating infection from flocks but can increase the threshold for infection, reduce the level of excretion of the organism and reduce vertical transmission in poultry that results in contamination of hatching or table eggs. Vaccination is therefore an aid to other eradication and control measures such as culling, all in-all out production, biosecurity and farm hygiene.

iii) Stability

Information is lacking on the stability of killed vaccines. Stability is affected by storage conditions and by the presence of contaminating microorganisms growing in the product. Chemicals with antimicrobial activity, such as thiomersal, phenol or crystal violet, are often included as preservatives in killed bacterial vaccines. The stability is assessed by potency tests repeated at appropriate time intervals. The stability of live vaccines can be assessed by performing counts of the number of viable organisms repeated at appropriate time intervals, and genotyping tests to identify genetic changes during fermentation production.

3. Vaccines based on biotechnology

a) Vaccines available and their advantages

None commercially available.

b) Special requirements for biotechnological vaccines, if any

Not applicable.

~~**b) Method of culture**~~

~~*Salmonella* Gallinarum is grown on or in a suitable medium, such as nutrient agar or broth, for 24 hours at 37°C.~~

~~**c) Validation as a vaccine**~~

~~There is no satisfactory method of assessing the protection afforded by the vaccine in the field. However, experience has shown that the vaccine can provide some benefit in some situations where control cannot be achieved by hygiene and management alone. The potency test described below may be used to provide evidence of efficacy.~~

2. Method of manufacture

The vaccine may be prepared by inoculation of a suitable medium, such as nutrient broth, with a fresh culture of *S.* Gallinarum (9R) and incubation at 37°C for 24 hours, with or without aeration. The organisms are harvested by sedimentation or centrifugation.

Alternatively the organisms may be grown on and harvested from a solid medium, such as nutrient agar. In either case, the suspension is diluted in PBS solution, pH 7.0, and may be freeze-dried. The dose used per bird is between 5×10^6 and 5×10^7 organisms.

3. In-process control

The culture used for inoculation of the production cultures and the harvested cells are examined microscopically using Gram staining to check for purity.

4. Batch control

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.9.

b) Safety

Six healthy, susceptible (preferably specific pathogen free [SPF]) chickens, 8–16 weeks of age, are each injected subcutaneously with ten doses of vaccine, and are observed for at least 7 days; no local or systemic reaction should develop.

c) Potency

Fifteen healthy chickens, 8–16 weeks of age, of the Light Sussex or Rhode Island Red breeds, or crosses of these, and taken from a stock that is free from *S.* Pullorum infection, are each injected subcutaneously with a quantity of vaccine corresponding to one field dose, i.e. 5×10^7 viable organisms. After an interval of 21–28 days, the vaccinated chickens and an equal number of similar unvaccinated chickens are deprived of food for approximately 18 hours. The chickens are then challenged by oral administration of 1 ml of a broth suspension containing 5×10^7 organisms of a virulent strain of *S.* Gallinarum mixed with 300 mg of a powder consisting of chalk (40%), light kaolin (43%) and magnesium trisilicate (17%). All the chickens are observed for 14–21 days. The vaccine passes the test if at the end of this period the number of surviving vaccinated chickens that show no macroscopic lesions of fowl typhoid at post-mortem exceeds by eight or more the number of similarly defined control chickens.

d) Duration of immunity

The vaccine should provide protection throughout the laying period, and this can be measured by potency (efficacy) tests at stages during lay. A booster dose during lay may be required, but should not be used during lay in flocks providing eggs for human consumption.

e) Stability

The shelf life of the vaccine can be measured by conducting potency tests at periods after manufacture. These should be done on at least six samples. Potency should remain satisfactory for at least 1 year.

f) Preservatives

No preservatives are used.

g) Precautions (hazards)

The organism is not known to be pathogenic to humans, and there are no special risks associated with the manufacture of either the vaccine or the antigen. However, the vaccine may establish a persistent infection in carrier birds and can precipitate disease in already infected chickens.

5. Tests on the final product

a) Safety

The safety test described in Section C.4.b should be used on a representative sample from each batch of final product.

b) Potency

~~The potency test described in Section C.4.c should be used on a representative sample from each batch of final product.~~

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ADVICE FOR MEMBER COMMENTS

Chapter Number and Title: 2.5.2. Contagious equine metritis

Country making the comments

Date

It would be appreciated if the following guidance notes are followed in making a reply:

1. *Comments may be general or specific, but specific comments are usually more valuable. General comments should be such that some conclusion and action can be taken in response to them. For example, it is of little help to make statements such as "This test is no longer used in our laboratory", without indicating the reasons and what test is used instead.*

2. *Specific comments should be identified, by indicating the line number in the text, to facilitate the making of editorial changes.*

3. *The pointing out of typing or technical errors is welcome, but the correct word or figure should be indicated. For example, it is of little help to say that 0.8 M is too high, if the preferred value is not indicated.*

4. *Bear in mind that the introductory chapters are not intended to be exhaustive, and indeed none of the chapters can give a completely comprehensive cover of the subject, otherwise the* Terrestrial Manual *would be too long. However, assistance in indicating priorities is always helpful.*

5. *The* Terrestrial Manual *is intended for world-wide use. The chapters need to reflect the development of new technology, but the old established methods, usually requiring less sophisticated apparatus, have to be retained. New technology should not be described in detail until it has gained wide acceptance as a reliable method.*

General Comments

Specific Comments (add continuation sheets if required)
line:

CHAPTER 2.5.2.

CONTAGIOUS EQUINE METRITIS

SUMMARY

Contagious equine metritis is an inflammation of the endometrium of mares caused by Taylorella equigenitalis*, which usually results in temporary infertility. It is a nonsystemic infection, the effects of which are restricted to the reproductive tract of the mare.*

~~If present,~~ chief clinical signs are a slight to copious mucopurulent vaginal discharge and a variable cervicitis and vaginitis. Recovery is uneventful, but prolonged asymptomatic carriage is established in a proportion of infected mares. ~~Taylorella~~ *Direct venereal contact between infected animals during natural mating presents the highest risk for the transmission of* T. equigenitalis*. Indirectly, the infection may be acquired through fomite transmission, manual cross contamination, inadequate obstetric hygiene at artificial insemination (AI) and using semen from* ~~equigenitalis *is most frequently transmitted by sexual contact with*~~ *carrier stallions~~, which~~. Carrier stallions are always ~~asymptomatic~~ subclinically infected and ~~in which~~ the principal sites of* T. equigenitalis *colonisation are the urogenital membranes (urethral fossa, urethral sinus, urethra and penile sheath). ~~Inadequate hygiene during the cleansing or examination of the genitalia of horses can also be responsible for the transmission of infection.~~ The sites of persistence of T. equigenitalis in the mare are urogenital membranes, principally in the clitoral sinuses and fossa and very infrequently in the uterus. Foals born of carrier mares may also become carriers. The organism can infect equid species other than horses, e.g. donkeys.*

~~Washing with disinfectants combined with local and systemic antibiotic treatment can eliminate~~ T. equigenitalis*~~. Vaccination has been found to be ineffective.~~ The principal means of control is through preventing transmission by establishing that stallions and mares are free from* T. equigenitalis *before breeding commences. Determination of the carrier state depends on the detection of* T. equigenitalis *on urogenital swabs of mares and stallions and its accurate identification. ~~Serum antibody to~~* T. equigenitalis *~~can be detected in mares for 3–7 weeks after infection and can also be demonstrated in the occasional carrier mare, but never in the stallion. Serology is of value in detecting recent, but not chronic, infection in the mare, but the emphasis for control of the disease should be on the detection of carriers by culture~~. Washing with antiseptics combined with local and systemic antibiotic treatment can eliminate* T. equigenitalis*.*

Identification of the agent: *~~Swabs must be transported to the laboratory with precautions~~ To avoid loss of viability swabs should be fully submerged in Amies charcoal medium and transported to the testing laboratory ~~preferably~~ in temperature-controlled conditions for plating out within 48 hours of collection. Growth of* T. equigenitalis *is likely to take ~~at least 72 hours~~ 3–6 days and may take up to 14 days, but usually does not take longer than 6 days at 37°C on ~~medium enriched with heated blood and~~ specialised media in an atmosphere of 5–10% CO_2. An incubation time of at least 7 days is advisable before certifying cultures negative for* T. equigenitalis*. ~~After 72 hours under appropriate culture conditions colonies may be small – up to 2–3 mm in diameter – watery to opaque and yellowish grey, and are smooth with an entire edge.~~* Taylorella equigenitalis *~~is a Gram-negative, small, coccoid rod that is sometimes pleomorphic and exhibits bipolar staining. It produces catalase and phosphatase, and is strongly oxidase positive. It is otherwise unreactive biochemically, and identification is finally dependent on antigenic characterisation of an isolate using specific antibodies.~~ Identification may include antigenic characterisation using specific antibodies and molecular genotyping. The fastidious nature of* T. equigenitalis *makes it difficult to isolate and test-breeding of stallions for detection of the carrier state has been used as a valuable adjunct to cultural examination.*

~~*Recently*~~ *Another species of* Taylorella*,* T. asinigenitalis, *has been isolated from male donkeys and horse stallions in the United States of America and stallions in Europe. This bacterium has not been associated with naturally occurring disease; it resides in the genital tract of male donkeys and can be passed to other donkeys and horses during mating.*

Antibody to whole killed T. equigenitalis *and a latex agglutination kit employing these antibodies may be used. Specificity for the organism and evidence of its failure to react with other* ~~*oxidase positive/catalase positive, Gram-negative*~~ *bacteria that might be cultured from the urogenital tract of horses is essential.* ~~*Monoclonal antibodies have been developed that can be used successfully to identify*~~ T. equigenitalis ~~*and distinguish it from strains of* T. asinigenitalis.~~

Serological tests: *Serology is of value in detecting recent, but not chronic, infection in the mare and serum antibody to* T. equigenitalis *can be detected in mares for 3–7 weeks after infection. It may also be demonstrated in the occasional carrier mare, but never in the stallion. No individual serological test described to date* ~~*will, by itself,*~~ *has reliably detected infection for diagnosis and control purposes. Serological tests can be used as an adjunct to culture for* T. equigenitalis *in screening mares recently bred to a carrier stallion, but must not be used as a substitute for culture.*

Requirements for vaccines ~~***and diagnostic biologicals:***~~ *Effective vaccines* ~~*that protect against contagious equine metritis or prevent colonisation by*~~ T. equigenitalis ~~*are not yet available*~~.

A. INTRODUCTION

Contagious equine metritis was first described in the United Kingdom (UK) in 1977 (Crowhurst, 1977), after which it was diagnosed in a number of countries world-wide. It first presented as disease outbreaks characterised by a mucopurulent vaginal discharge originating from inflammation of the endometrium and cervix, resulting in temporary infertility. The fastidious nature and slow growth of the causative bacterium, *Taylorella equigenitalis*, caused difficulties in initial attempts at culture (Platt *et al*., 1977), but the disease was reproduced by experimental clitoral challenge with isolated laboratory-grown bacteria (Platt *et al.,* 1978; Swaney & Sahu 1978). Using appropriate culture conditions, *T. equigenitalis* can be isolated from infective vaginal discharge. Mares may encounter more than one episode of the disease in a short period of time (Timoney *et al.,* 1977). Serum antibody persists for 3–7 weeks after infection, but often it is not detectable for up to 15–21 days after recovery from acute infection in the mares (Dawson *et al.,* 1978). Most mares recover uneventfully, but some may become carriers of *T. equigenitalis* for many months (Platt *et al.,* 1978). Colonisation by *T. equigenitalis* is most consistently demonstrated by culture of swabs taken from the recesses of the clitoral fossa and sinuses, but it may be recovered from the cervix and endometrium in pure culture (Platt *et al.,* 1978). Carriage does not always affect conception (Timoney *et al.,* 1978), and in such cases pregnancy may proceed so that foals are born, become infected by passage down the birth canal, and thereby also become long-term and subclinical carriers (Timoney & Powell, 1982). Many primary cases of infection with *T. equigenitalis* in the mare are subclinical, and a frequent indicator of infection is a mare returning in oestrus prematurely after being bred to a putative carrier stallion.

Carrier mares and stallions act as reservoirs of *T. equigenitalis*, but stallions, because they mate with numerous mares, play a much more important role in transmission of the bacterium. The urogenital membranes of stallions become contaminated at coitus, leading to a carrier state that may persist for many months or years (Schluter *et al.,* 1991). Unhygienic examination of mares and unsanitary washing of the stallion's penis may also spread the organism. Other sites of the horse's body are not known to harbour *T. equigenitalis*. Most carrier mares are clitoral carriers of *T. equigenitalis*. Long-term persistence of the organism in the uterus, though uncommon, can occur. To detect such carriers of *T. equigenitalis,* cervical or endometrial swab samples should be taken routinely in addition to sampling the clitoral area of all mares. *Taylorella equigenitalis* can cause abortion in the mare but this is a rare occurrence.

Taylorella equigenitalis is not known to infect humans and it should be handled in the laboratory at biosafety level 2.

~~B. DIAGNOSTIC TECHNIQUES~~

Disease control

Prior infection and vaccination are not fully protective (Fernie *et al.,* 1980), and failure of antibody to persist has meant that control of infection has relied entirely on prevention of transmission through the detection of *T. equigenitalis* on swabs of urogenital membranes. In spite of difficulties in culturing *T. equigenitalis*, screening mares and stallions prior to and while on the stud farm has successfully eliminated the disease from thoroughbred horses in countries using a voluntary code of practice. These have been based on the widely adopted UK's

Horserace Betting Levy Board's Code of Practice (www.hblb.org.uk/codes.htm), which is reviewed and updated annually ~~and updated as necessary~~ and will reflect current information. The key recommendations of the code are summarised below.

At the start of the breeding season, swabs are taken from all stallions, including those in their first breeding season, from the urethra, urethral fossa and sinus, penile sheath and pre-ejaculatory fluid, on two occasions no fewer than 7 days apart. Mares are classified according to the degree of risk that they represent, and the frequency of sampling is adjusted accordingly. High-risk mares are defined as: (a) those from which *T. equigenitalis* has been isolated (the high risk status will remain until three sets of negative swabs have been taken at three different oestrous periods in each of 2 years); (b) mares that have visited any premises on which *T. equigenitalis* has been isolated within the previous 12 months; (c) mares arriving from ~~Canada, France, Germany, Ireland, Italy, the UK and the USA that have been mated during the last breeding season with stallions resident outside these~~ countries currently listed in the guidelines as higher risk; d) all mares that have been in countries ~~other than Canada, France, Germany, Ireland, Italy, the UK and the USA~~ that are currently listed in the guidelines as higher risk within the last 12 months. ~~'Low risk' mares are any mares not defined as 'high risk'.~~

High risk stallions are defined as: (a) stallions that have not previously been used for breeding purposes; (b) stallions from which *T equigenitalis* has been isolated (the 'high risk' status will remain until treatment has been undertaken and required swab results are negative); (c) stallions that have been, within the last 12 months, on any premises on which *T equigenitalis* has been isolated; (d) stallions that have mated a mare which has not been swabbed negative in accordance with the Code of Practice. 'Low risk' stallions are any stallions not defined as 'high risk'. There is a potentially serious problem with contagious equine metritis in this group of horses, especially those belonging to non-thoroughbred breeds.

Results of laboratory tests for *T. equigenitalis* should be entered on an officially approved certificate, which is sent to the veterinarians and stallion stud farm managers who supervise the breeding. The certificate should record the animal's name, the sites and date of swabbing, the name of the veterinarian taking the swabs, identity of the testing laboratory, the date the swabs were received and cultured by the laboratory, and whether the swabs were negative or positive, or whether the culture was overgrown by other bacteria to an extent that the laboratory could not be confident that small numbers of *T. equigenitalis* would be detected and, therefore, requiring another set of swabs to be collected.

Difficulties with the culture of *T. equigenitalis* caused by its fastidious nature necessitate the use of a quality control system that should be approved before a laboratory is permitted to undertake official testing for contagious equine metritis and to issue certificates of the test results. The task of quality control should be undertaken by an experienced, reliable, and impartial microbiology laboratory~~,~~ authorised for the purpose. ~~which is not involved in routine testing of contagious equine metritis swabs. At 6-month intervals, swabs inoculated into mixed cultures that are designed to test a laboratory's ability to recover and identify *T. equigenitalis* in the face of contaminants, as well as procedures for reporting results, should be sent to laboratories wishing to be approved for testing for this bacterium. A list of those laboratories satisfactorily passing the quality control should be published in a veterinary journal that is widely read by the national veterinarians. The veterinarians and stallion stud farm managers who supervise the breeding mares and stallions should accept only certificates provided by laboratories currently approved for that purpose~~

Any mares with abnormal vaginal exudate, or returning to oestrus prematurely, should be investigated and managed as though infected with *T. equigenitalis* until results of laboratory testing prove otherwise. Other causes of outbreaks of endometritis include *Pseudomonas aeruginosa*, *Streptococcus zooepidemicus* and certain capsule types of *Klebsiella pneumoniae*. Swabs should be examined for these bacteria, and an attempt should be made to culture and identify *K. pneumoniae* and *P. aeruginosa* so as to establish a differential diagnosis.

If carriers of *T. equigenitalis* are detected, the organism can be eliminated by treatment with systemic antibiotics combined with ~~disinfectant~~ antiseptic washing of exposed genital membranes ~~(1)~~. Particular attention should be paid to cleansing of the recesses of the clitoral fossa and sinuses of mares, where colonisation by *T. equigenitalis* is frequently found in carrier animals. A course of treatment may take several weeks and may need to be repeated before intensive swabbing consistently fails to recover *T. equigenitalis* in stallions and mares (Crowhurst *et al.,* 1979). A significant number of carrier mares can be refractive to several courses of treatment. These may require surgery and ablation of the clitoral sinuses for permanent elimination of the carrier state in such animals.

Control measures for countries regarded as free from *T. equigenitalis* infection should be based on the screening of animals prior to importation and/or during a post-importation quarantine period using swabbing and testing regimes broadly based on those described above for breeding populations.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent (the prescribed test for international trade)

Various bacteria may be present on the urogenital membranes of horses as harmless commensals and may interfere with the culture of *T. equigenitalis*. Some may initially be present in small numbers, but multiply on the swab before it is cultured. Overgrowth of these organisms on the culture plates may obscure the presence of *T. equigenitalis*. Swabs must be placed in a transport medium with activated charcoal, such as Amies medium, to absorb inhibitory by-products of bacterial metabolism (Swerczek, 1978). Over time, numbers of *T. equigenitalis* decline on swabs with time, and this effect is more pronounced at higher temperatures (Sahu *et al.,* 1979). Swabs must be kept cool during transportation and should arrive at the laboratory no later than 24–48 hours after they were taken. Negative culture results from swabs plated out more than 48 hours after they were taken are unreliable. Antibiotic treatment for whatever cause should cease at least 7 days before swabbing. The presence of antibiotics may sublethally damage *T. equigenitalis,* which nonetheless persists on the urogenital membranes but cannot be grown on laboratory media.

Each swab must be inoculated on to 5% (v/v) heated blood, ('chocolate'), agar plates, produced by heating the liquid medium, containing blood, at 70–80°C for 12 minutes. When cooled to 45–50°C, trimethoprim (1 µg/ml), clindamycin (5 µg/ml), and amphotericin B (5–15 µg/ml), is added to the medium, Timoney *et al.* (1982). Thymidine, which will inactivate trimethoprim, is present in bacteriological media containing peptone, so it is important to add 5% lysed horse blood at this stage. Lysed horse blood contains thymidine phosphorylase, which will inactivate thymidine, thus allowing the trimethoprim to exert its selective effect. This is the preferred medium for isolating *T. equigenitalis;* this medium has been used successfully to isolate equally well both biotypes of this pathogen and to eliminate the growth of many commensal bacteria and inhibit fungal growth. As inhibiters may prevent the isolation of some strains of *T. equigenitalis*, swabs should also be inoculated on to plates of 5% 'chocolate' blood agar with a rich peptone agar base containing additional cysteine (0.83 mM), sodium sulphite (1.59 mM) and a fungicide (5–15 µg/ml amphotericin B). *Taylorella equigenitalis* will grow on blood agar, but it can tolerate less than ideal conditions better when grown on 'chocolate' blood agar as described above. Some manufacturers[1] produce a peptone agar base that is quality controlled for its ability to support the growth of *T. equigenitalis*. The quality of the commercial agar should be confirmed by the testing laboratory. An important feature of all good *T. equigenitalis* media is the absence of fermentable carbohydrates. These do not enhance the growth of *T. equigenitalis*, but their fermentation by other bacteria inhibits *T. equigenitalis* growth (Atherton, 1983; Fernie *et al.,* 1980). A third medium containing streptomycin sulphate (200 µg/ml) is sometimes used as some isolates of *T. equigenitalis* are resistant to this concentration of antibiotic, which serves to reduce the extent of growth of other bacteria that might otherwise obscure the presence of small numbers of *T. equigenitalis* (Swerczek, 1978). However, a streptomycin-sensitive biotype is now the most common strain isolated and will not be detected on this medium; consequently, it should only be used in conjunction with medium without streptomycin. Growth by other bacteria, for example *Proteus mirabilis*, however, may be so extensive that the laboratory should record that they cannot issue a negative result. In this event, further swabs should be requested in the hope that the problem will not recur.

Occasionally, the urogenital membranes of stallions or mares will be persistently colonised by another bacterium that interferes with diagnosis, and it will prove necessary to attempt to eliminate this by washing and antibiotic treatment. Swabbing for *T. equigenitalis* shall not recommence until at least 7 days after treatment has stopped. The use of Timoney's medium (Timoney *et al.,* 1982), described above, should overcome this difficulty in most cases

All culture media prepared should be subjected to quality control and must support growth of the suspect organism from a small inoculum, before their use on suspect samples. The reference strain of *T. equigenitalis* must also be cultured in parallel with the test samples to ensure that the culture conditions are optimal for isolation of this organism. It is important to emphasise that alongside each day's tests, additional plates should be inoculated with a culture of *T. equigenitalis* to check that each batch of medium will support growth.

The fastidious nature of *T. equigenitalis* makes it difficult to isolate. Test breeding of stallions has been used to increase the sensitivity of detection of the carrier state and it serves as a valuable adjunct to cultural examination. The numbers of *Taylorella* mechanically carried by stallions can be very low and may be missed by culturing swabs, but can be detected after multiplication in the mare that has been test bred. The use of test breeding as a diagnostic tool can be especially important in countries that are considered free from contagious equine metritis.

~~Occasionally, the urogenital membranes of stallions or mares will be persistently colonised by another bacterium that interferes with diagnosis, and it will prove necessary to attempt to eliminate this by washing and antibiotic treatment. Swabbing for *T. equigenitalis* shall not recommence until at least 7 days after treatment has stopped. The use of Timoney's medium (31), described above, should overcome this difficulty in most cases. It is important~~

1 For example, Mast Diagnostics, Mast House, Derby Road, Bootle, Merseyside L20 1EA, United Kingdom (UK), and Lab M, Tomley House, Wash Lane, Bury BL9 6AU, UK.

~~to emphasise that alongside each day's tests, additional plates should be inoculated with a culture of *T. equigenitalis* to check that each batch of medium will support growth.~~

Plates must be incubated at 35–37°C in 5–10% (v/v) CO_2 in air or by use of a candle jar. At least 72 hours is normally required before colonies of *T. equigenitalis* become visible, after which time daily inspection is needed. Visual detection of colonies may take up to 14 days (Ward *et al.,* 1984). A standard incubation time of at least 7 days is advisable before certifying cultures negative for *T. equigenitalis*. Plates should be examined for contaminants after the first 24 hours' incubation. Colonies of *T. equigenitalis* may be up to 2–3 mm in diameter, smooth with an entire edge, glossy and yellowish grey. Laboratories should be aware that certain countries require the prolonged incubation period as a standard procedure and should therefore ascertain the particular import requirements and/or indicate the incubation period on which their cultural findings are based.

Taylorella equigenitalis is a Gram-negative, nonmotile, bacillus or cocco-bacillus that is often pleomorphic (up to 6 µm long) and may exhibit bipolar staining. It is catalase positive, phosphatase positive, and strongly oxidase positive ~~(see ref. 5 for methods for examining catalase, phosphatase and oxidase activities)~~. It is otherwise inert in tests for biochemical activity. If a slow-growing organism is isolated that fits the description for cellular morphology and that is strongly oxidase positive, it should be tested for reactivity with *T.-equigenitalis*-specific antiserum.

A variety of serotyping tests have been developed ranging in complexity from slide agglutination to direct or indirect immunofluorescence. Each method has its advantages and disadvantages. The disadvantage of the slide agglutination test is that occasionally autoagglutination of isolates occurs: culturing in bottled CO_2 in air, as opposed to in a candle jar, may reduce autoagglutination (Ter Laak & Wagenaars, 1990). It has been suggested that immunofluorescence can be used to identify autoagglutinating isolates, some workers have reported cross-reaction with *Mannheimia haemolytica* but this is very rare. If a cross-reaction is suspected, it may be necessary to repeat the test using adsorbed antisera (Ter Laak & Wagenaars, 1990). The specificity of the immunofluorescence test can be improved by the use of monoclonal antibodies that are now available and have been used in the differentiation of *T. equigenitalis* and *T. asinigenitalis* (Breuil *et al.,* 2010) [2].

Antiserum is produced by vaccinating rabbits with killed *T. equigenitalis*. A number of different immunisation regimes can be employed, ranging from those used for producing *Escherichia-coli*-typing antisera ~~(26)~~ to immunisation together with an adjuvant, such as Freund's incomplete. Monoclonal antibodies are available commercially that provide a highly specific means of identifying *T. equigenitalis*. A standard strain, such as NCTC 11184[3], should be used for immunisation. However, the most important consideration is the specificity of the antiserum produced. It should agglutinate *T. equigenitalis*, but fail to agglutinate other bacteria that might be cultured from horse urogenital membranes, even if rarely. In particular, it should not agglutinate any oxidase-positive and Gram-negative rods, such as *Mannheimia haemolytica*, *Actinobacillus equuli, Bordetella bronchiseptica* (to which *T. equigenitalis* is closely related, see Bleumink-Pluym *et al.* (1993), and *Pseudomonas aeruginosa*. Recently another species of *Taylorella*, *T. asinigenitalis,* has been isolated from male donkeys in the United States of America (Jang *et al.,* 2001) and from a horse stallion in Europe (Baverud *et al.,* 2006). This newly described bacterium, which has not been associated with naturally occurring disease, resides in the genital tract of male donkeys and can be passed to donkeys and horses during mating. Moreover it has similar, though not identical, colonial appearance and cultural characteristics and gives identical biochemical test results to those used to confirm the identity of *T. equigenitalis*. There is even serological cross-reactivity between the two organisms. ~~At the National Veterinary Services Laboratories (NVSL) in Ames, Iowa, USA[4], and at the Veterinary Laboratories Agency (VLA), Bury St Edmunds, United Kingdom[4],~~ Differentiation of *T. asinigenitalis* from *T. equigenitalis* is possible using the polymerase chain reaction (PCR) or 16S rDNA sequencing and biochemical reactivity (Baverud *et al.,* 2006; Wakeley *et al.,* 2006).

A latex agglutination kit is available commercially for the antigenic identification of *T. equigenitalis*. It is based on polyclonal antibodies produced using methods similar to those described above. This is widely used by routine testing laboratories for the confirmation of the identity of colonies growing on selective medium that give a biochemical reaction consistent with *T. equigenitalis*. As *T. equigenitalis* is antigenically relatively distinct, and small amounts of cross-reactive antibody are easily absorbed during production of the reagent, the test has proved to be highly specific and sensitive. It should be emphasised that it will not necessarily distinguish strains of *T. equigenitalis* from *T. asinigenitalis.*

Molecular testing methods such as PCR and real time PCR (rtPCR) have been applied to the detection of *T.equigenitalis* both directly (using swabs taken from sampling sites) and indirectly (from cultures grown from swabs) (Bleumink-Pluym *et al.,* 1994). ~~A PCR method has been used for detecting *T. equigenitalis* and was compared with culture methods in the Netherlands and Japan (2, 7, 10). In these studies a much higher rate of~~

2 Institut Pourquier, 326 rue de la Galera, Parc Euromedicine, 34090 Montpelier, France. E-mail: info@institut-pourquier.fr

3 Obtainable from the National Collection of Type Cultures, Colindale, London, UK.

~~4 For further information contact the NVSL at NVSL, P.O. Box 844, Ames, Iowa 50010, USA. E-mail: NVSL_Concerns@aphis.usda.gov or the VLA at VLA, Rougham Hill, Bury St Edmunds, IP33 2RX, UK. E-mail: bury-st-edmunds@vla.defra.gsi.gov.uk~~

~~detection by PCR was found than by culture, even among horses imported from a source without previous evidence of *T. equigenitalis* infection or clinical disease. The authors proposed that carriage is more widespread than previously believed, and that recently discovered genetic variation among strains (8, 18) may relate to differences in pathogenicity. The PCR has also been used in the UK (11).~~ In studies carried out in the UK it was shown to be highly specific and was able to detect very small numbers of *T. equigenitalis* in the presence of very large numbers of bacteria comprising the background flora harvested from culture plates inoculated with samples of the equine urogenital tract (Dawson *et al.,* 1978). ~~Recently~~ In Japan the field application of the PCR in the eradication of contagious equine metritis was evaluated. It was demonstrated that the PCR was more sensitive than culture for the detection of *T. equigenitalis* from genital swabs of horses ~~in the field~~ (Anzai *et al.,* 2002; Moore *et al.,* 2001). A real-time PCR was developed in the UK for use directly on genital swabs and compared with culture (Wakeley *et al.,* 2006) and this has been subsequently used for pre-breeding screening studies (Ousey *et al.,* 2009). There was no significant difference in the performance of the direct PCR and culture, but the PCR had the added advantage of speed of result and also differentiated *T.* equigenitalis from *T. asinigenitalis*. ~~This promising technique needs to be more fully and widely evaluated, especially~~ Commercial PCR kits are available for the detection of *T. equigenitalis* and these may be used to enhance the testing capabilities of authorised laboratories.

2. Serological tests

No serological test described to date will, by itself, reliably detect infection for diagnosis and control. However, the complement fixation test has been used successfully as an adjunct to culture for *T. equigenitalis* in screening mares between 21 and 45 days after being bred to suspect a carrier stallion.

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

Effective vaccines that protect against contagious equine metritis or prevent colonisation by *T. equigenitalis* are not yet available.

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* *

NB: There are OIE Reference Laboratories for Contagious equine metritis (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).

ADVICE FOR MEMBER COMMENTS

Chapter Number and Title: 2.7.7. Enzootic abortion of ewes (ovine chlamydiosis)

Country making the comments

Date

It would be appreciated if the following guidance notes are followed in making a reply:

1. *Comments may be general or specific, but specific comments are usually more valuable. General comments should be such that some conclusion and action can be taken in response to them. For example, it is of little help to make statements such as "This test is no longer used in our laboratory", without indicating the reasons and what test is used instead.*

2. *Specific comments should be identified, by indicating the line number in the text, to facilitate the making of editorial changes.*

3. *The pointing out of typing or technical errors is welcome, but the correct word or figure should be indicated. For example, it is of little help to say that 0.8 M is too high, if the preferred value is not indicated.*

4. *Bear in mind that the introductory chapters are not intended to be exhaustive, and indeed none of the chapters can give a completely comprehensive cover of the subject, otherwise the* Terrestrial Manual *would be too long. However, assistance in indicating priorities is always helpful.*

5. *The* Terrestrial Manual *is intended for world-wide use. The chapters need to reflect the development of new technology, but the old established methods, usually requiring less sophisticated apparatus, have to be retained. New technology should not be described in detail until it has gained wide acceptance as a reliable method.*

General Comments

Specific Comments (*add continuation sheets if required*)
line:

CHAPTER 2.7.7.

ENZOOTIC ABORTION OF EWES
(ovine chlamydiosis)

SUMMARY

Ovine chlamydiosis, also known as enzootic abortion of ewes (*EAE*) *or ovine enzootic abortion* (*OEA*), *is caused by the bacterium* ~~Chlamydophila~~ Chlamydia abortus. *Chlamydial abortion typically occurs in the last 2–3 weeks of pregnancy with the appearance of stillborn lambs and inflamed placentas. However, infection can also result in the delivery of full-term stillborn lambs or weak lambs that do not survive longer than 48 hours. Infected ewes can also give birth to healthy lambs and it is not uncommon to observe delivery of a dead and a weak or healthy lamb. There are rarely any predictive signs that abortion is going to occur, although behavioural changes and a vulval discharge can be observed in the last 48 hours of pregnancy.*

Diagnosis of enzootic abortion depends on the isolation and identification of the causative agent or detection of the agent or its nucleic acid in the products of abortion or vaginal excretions of freshly aborted females. A humoral antibody response may be detected following abortion. Goats as well as sheep and, less commonly, cattle, pigs, horses and deer, can be affected. Chlamydiosis of small ruminants is a zoonosis and the organism must be handled with biosafety precautions. Pregnant women are particularly at risk.

Identification of the agent: *The basis for a positive diagnosis of infection with* C. abortus *depends on a history of abortion in sheep or goats* (*often in late pregnancy*), *evidence of necrotic placentitis, and the demonstration of large numbers of the organism in stained smears of affected placentae. The still moist fleece of fetuses or vaginal swabs of females that have freshly aborted are also useful. Care is needed to distinguish cotyledonary damage caused by* Toxoplasma gondii *and, in stained smears, to be aware of the morphological similarities between* C. abortus *and* Coxiella burnetii, *the agent of Q fever.*

Chlamydial antigen can be detected by enzyme-linked immunosorbent assay, immuno-histochemistry or the fluorescent antibody test, whereas chlamydial DNA can be detected by the polymerase chain reaction or by microarray. Some of these methods are available in commercial kit form.

~~Chlamydophila~~ Chlamydia abortus *can be isolated only in living cells; thus facilities for growth in chicken embryos or cell cultures, with appropriate biohazard containment, are required.*

Serological tests: *A rise in antibody titre to* C. abortus*, detected by the complement fixation* (*CF*) *test, is common after abortion or stillbirth, but this does not occur in every case.* ~~Chlamydophila~~ Chlamydia abortus *shares common antigens with* C. pecorum *and some Gram-negative bacteria, so that the CF test is not wholly specific, nor does it distinguish between responses to vaccination and to infection. Low CF titres need to be interpreted with caution, particularly if these are encountered in individual animals or in flocks with no history of abortion.*

Alternative serological tests have been developed and some commercialised, but none has been sufficiently appraised so far for field use. A delayed hypersensitivity reaction to chlamydial antigen can be elicited in infected sheep, but the procedure is not amenable to routine use.

Requirements for vaccines and diagnostic biologicals: *Inactivated and live vaccines are available that have been reported to prevent abortion and to reduce excretion. They assist in control of the disease but will not eradicate it. Serological screening during the period after parturition helps to identify infected flocks, to which control measures can then be applied.*

A. INTRODUCTION

Ovine chlamydiosis (enzootic abortion of ewes [EAE] or ovine enzootic abortion [OEA]) is caused by the bacterium ~~*Chlamydophila*~~ *Chlamydia abortus.* Chlamydial abortion in late pregnancy causes serious reproductive wastage in many sheep-rearing areas of the world, particularly where flocks are closely congregated during the parturient period (Aitken, 2000; Longbottom & Coulter, 2003). Abortion typically occurs in the last 2–3 weeks of pregnancy with the appearance of stillborn lambs and grossly inflamed placentas. Infection can also result in the delivery of full-term stillborn lambs and weak lambs that generally fail to survive beyond 48 hours. It is also not uncommon in multiple births for an infected ewe to produce one dead lamb and one or more weak or healthy lambs. Infection is generally established in a 'clean' flock through the introduction of infected replacements and results in a small number of abortions in the first year, which is followed by an 'abortion storm' in the second year that can affect up to around 30% of ewes.

Infected animals show no clinical illness prior to abortion, although behavioural changes and a vulval discharge may be observed in ewes within the last 48 hours of pregnancy. Pathogenesis commences around day 90 of gestation coincident with a phase of rapid fetal growth when chlamydial invasion of placentomes produces a progressively diffuse inflammatory response, thrombotic vasculitis and tissue necrosis. Milder changes occur in the fetal liver and lung and, in cases in which placental damage is severe, there may be evidence of hypoxic brain damage (Buxton *et al*., 2002). Abortion probably results from a combination of impairment of materno-fetal nutrient and gaseous exchange, disruption of hormonal regulation of pregnancy and induced cytokine aggression (Entrican, 2002).

Chlamydial abortion also occurs in goats and, less frequently, cattle, pigs, horses and deer may be affected. In sheep, abortion in late pregnancy with expulsion of necrotic fetal membranes are key diagnostic indicators, with care being needed to distinguish the diffuse pattern of necrosis from that caused by *Toxoplasma gondii* (cotyledons only). Distinction from other infectious causes of abortion such as brucellosis (see Chapter 2.7.2), coxiellosis (see Chapter 2.1.12) or other bacterial pathogens (*Campylobacter* [see Chapter 2.9.3], *Listeria* [see Chapter 2.9.7], *Salmonella* [see Chapter 2.9.9]) can be achieved by microscopy and/or culture.

The taxonomy of the family *Chlamydiaceae* was again revised recently by abolishing the subdivision into the genera *Chlamydia* (*C.*) and *Chlamydophila* that had been introduced in 1999 (Everett *et al*., 1999). Now the recombined genus *Chlamydia* (Kuo *et al*.) includes all nine species, i.e. *C. trachomatis* (human), *C. suis* (swine), *C. muridarum* (mouse, hamster), *C. psittaci* (birds and others), *C. felis* (cats), *C. abortus* (sheep, goats, cattle), *C. caviae* (guinea pigs), and *C. pecorum* (sheep, cattle) and *C. pneumonia* (human and others). While most of these organisms are highly host specific, *C. pneumoniae* and *C. psittaci* have a broader host range. ~~Taxonomically, the family Chlamydiaceae is divided into two genera and nine species based on sequence analysis of the 16s and 23s rRNA genes (12). The genus *Chlamydia* includes *C. trachomatis* (humans), *C. suis* (swine) and *C. muridarum* (mouse and hamster). The genus *Chlamydophila* includes *C. psittaci* (avian), *C.felis* (cat), *C. abortus* (sheep, goat and cattle), *C. caviae* (guinea-pig), the former species *C. pecorum* (sheep and cattle) and *C. pneumoniae* (humans). The two genera and nine species have merit both on the basis of molecular structure and for the purposes of host range and clinical disease. The species show a marked degree of correlation with host range, disease syndrome and virulence, thus assisting in understanding the epidemiology of the various species and serovars affecting mammals and birds.~~ The terms 'chlamydiosis' and 'chlamydia(e)' are used to refer to members of ~~either of the two genera~~ the genus *Chlamydia* in general. However, a binomial of the generic and specific names is used when referring to a particular chlamydial species.

Infected females shed vast numbers of infective *C. abortus* at the time of abortion or parturition, particularly in the placenta and uterine discharges (Papp *et al*., 1994). Environmental contamination, resulting from this shedding, is considered the primary source of infection for other females. There is also limited evidence to suggest that sheep that have experienced an abortive episode following experimental infection, can also excrete *Chlamydia* from the reproductive tract during subsequent oestrus cycles (Papp *et al*., 1994). Human infection may be acquired from infected products of abortion or parturition or from carelessly handled laboratory cultures of the organism, with effects that range from subclinical infection to acute influenza-like illness. Appropriate precautions should be taken when handling cultures and potentially infected tissues (see Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities). Authenticated cases of human placentitis and abortion caused by *C. abortus* of ovine origin indicate that pregnant women are at special risk and should not be exposed to sources of infection (Caul & Sillis, 1998; Longbottom *et al*., 2002).

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

a) Smears

Where the clinical history of the flock and the character of lesions in aborted placentae suggest enzootic abortion, a diagnosis can be attempted by microscopic examination of smears made from affected chorionic

villi or adjacent chorion. Several staining procedures are satisfactory, for example, modified Machiavello, Giemsa, *Brucella* differential, or modified Ziehl–Neelsen stains (Stamp *et al*., 1950). In positive cases stained by the latter method and examined under a high-power microscope, large numbers of small (300 nm) coccoid elementary bodies are seen singly or in clumps stained red against the blue background of cellular debris. Under dark-ground illumination, the elementary bodies are pale green. If placental material is not available, smears may be made from vaginal swabs of females that have aborted within the previous 24 hours, or from the moist fleece of a freshly aborted or stillborn lamb that has not been cleaned by its mother. In general, such preparations contain fewer organisms than placental smears.

In terms of morphology and staining characteristics, *C. abortus* resembles the rickettsia *Coxiella burnetii,* which, in some circumstances, may provoke abortion and which, in humans, causes Q fever. Care must be taken to differentiate between these two organisms in cases lacking a good history or evidence of chlamydial-induced placental pathology. Antigenic differences between *C. abortus* and *Coxiella burnetii* can be detected serologically. Fluorescent antibody tests (FATs) using a specific antiserum or monoclonal antibody may be used for identification of *C. abortus* in smears.

b) Antigen detection

Several chlamydial genus-level antigen-detection tests are available commercially. A comparative assessment of several such assays, on non-ovine material, indicated that those using enzyme-linked immunosorbent assay (ELISA) methodology were more sensitive than kits employing a FAT (Wood & Timms, 1992). Under the test conditions used, a kit that detects chlamydial lipopolysaccharide (LPS) was judged to be the most sensitive of the rapid ELISA-based systems investigated. Though occasionally yielding false-positive results, particularly with avian faecal samples, the kit also gave satisfactory results with ovine placental samples (Wilsmore & Davidson, 1991). In histopathological sections, antigen detection can be performed using commercially available anti-*Chlamydia* antibodies directed against LPS or MOMP (major outer membrane protein) (Borel *et al*., 2006).

c) DNA

Amplification of chlamydial DNA by polymerase chain reaction (PCR) and real time PCR provide alternative approaches for verifying the presence of chlamydiae in biological samples without resorting to culture. PCR is highly sensitive for this purpose, but has the attendant risk of cross-contamination between samples, so appropriate measures must be taken to avoid this happening (see Chapter 1.1.7 Biotechnology in the diagnosis of infectious diseases and vaccine development). Another potential problem is in the production of false negatives resulting from PCR-inhibitory substances in the samples. Methods for discriminating between amplified DNA sequences originating from *C. abortus* and *C. pecorum* have been described (DeGraves *et al*., 2003; Everett & Andersen, 1999; Jee *et al*., 2004; Laroucau *et al*., 2001; Thiele *et al*., 1992). In the last few years, real-time PCR has become the preferred method in diagnostic laboratories for its rapidity, high throughput and ease of standardisation (Sachse *et al.,* 2009). ~~Such tests are beginning to be introduced into diagnostic laboratories throughout Europe.~~ Recently, DNA microarray hybridisation assays using the ArrayTube™ platform have been developed and hold much promise for the direct detection and identification of organisms from clinical samples (Borel *et al.,* 2008; Sachse *et al*., 2005).

d) Tissue sections

Intracellular chlamydial inclusions can be demonstrated by Giemsa staining of thin (≤4 µm) sections taken from target tissues that have been suitably fixed in fluids such as Bouin or Carnoy. More striking results can be obtained by immunological staining procedures. The direct immunoperoxidase method (Finlayson *et al*., 1985) is rapid and simple, while the method with streptavidin–biotin is more complex (Szeredi & Bacsadi, 2002). Electron microscopy can also be performed using negative contrast, to differentiate chlamydiae from *Coxiella burnetii*.

e) Isolation of the agent

~~*Chlamydophila*~~ *Chlamydia abortus* can be isolated in embryonated chicken eggs or in cell culture, the latter being the method of choice for isolation of new strains. The causative agent of chlamydosis is zoonotic (Longbottom *et al*., 2002) and thus isolation and identification procedures must be carried out under the appropriate containment level as described in Chapter 1.1.2.

Tissue samples, such as diseased cotyledons, placental membranes, fetal lung or liver, or vaginal swabs, that may be subject to any delay before isolation procedures begin, should be maintained in a suitable transport medium in the interim period. The most satisfactory medium is sucrose/phosphate/glutamate or SPG medium (sucrose [74.6 g/litre], KH_2PO_4 [0.512 g/litre], K_2HPO_4 [1.237 g/litre], L-glutamic acid [0.721 g/litre]) supplemented with 10% fetal bovine serum, antibiotic (streptomycin and gentamycin are suitable, but not penicillin), and a fungal inhibitor (Spencer & Johnson, 1983). A tissue:medium ratio of 1:10 is commonly employed. Alternatively, approximately 1 g of tissue is ground with sterile sand in 8 ml of transport medium.

Chicken embryos: Test samples are prepared as 10% suspensions in nutrient broth containing streptomycin (not penicillin) (200 µg/ml); 0.2 ml of suspension is inoculated into the yolk sac of 6–8-day old embryos, which are then further incubated at 37°C. Infected embryos die between 4 and 13 days after inoculation. Smears prepared from their vascularised yolk sac membranes reveal large numbers of elementary bodies.

Cell cultures: ~~Chlamydophila~~ Chlamydia abortus of ovine origin can be isolated in a variety of cell types, but McCoy, Buffalo Green Monkey (BGM) or baby hamster kidney (BHK) cells are most commonly used. For confirmatory diagnosis, cultured monolayers are suspended in growth medium at a concentration of 2×10^5 cells/ml. Aliquots of 2 ml of the suspension are dispensed into flat-bottomed glass Universal bottles, each containing a single 16 mm cover-slip. Confluent cover-slip monolayers are achieved after incubation for 24 hours at 37°C. The growth medium is removed and replaced by 2 ml of test inoculum, which is then centrifuged at 2500 ***g*** for 30 minutes on to the cover-slip monolayer to promote infection. After further incubation for 2–3 days, the cover-slip monolayers are fixed in methanol and stained with Giemsa or according to the method of Gimenez (Arens & Weingarten, 1981; Gimenez, 1964). After methanol fixation, infected cultures contain basophilic (Giemsa) or eosinophilic (Gimenez) intracytoplasmic inclusions. Similar procedures are used in culturing *C. abortus* for antigen preparation. FAT techniques can also be used and are equally effective.

Chlamydial activity can be further enhanced by chemical treatment of cultured cells, before or during infection, to favour chlamydial growth. Various substances that have been described for incorporation into the infective inoculum to which cover-slip monolayers are exposed include: cycloheximide (0.5 µg/ml) in the maintenance medium, emetine (1 µg/ml) for 5 minutes before infection, and 5-iodo-2-deoxyuridine (80 µg/ml) for 3 days prior to infection. Unless preconditioned cells are available, the latter isolation procedure requires increased time for successful agent isolation.

2. Serological tests

a) Complement fixation test

Complement fixation (CF) is the most widely used procedure for detecting infection (sheep and goats are generally tested within 3 months of abortion or parturition). The test will also detect evidence of vaccination. Infection is evident principally during active placental infection in the last month of gestation and following the bacteraemia that often accompanies abortion. Consequently, paired sera collected at the time of abortion and again at least 3 weeks later may reveal a rising CF antibody titre that will provide a basis for a retrospective diagnosis. Antigenic cross-reactivity between *C. abortus* and *C. pecorum,* as well as with some Gram-negative bacteria (e.g. *Acinetobacter*), can give rise to low false-positive CF test results. Thus, titres less than 1/32 in individual animals should be considered to be nonspecific for *C. abortus,* although they could also be due to a low grade infection with *C. abortus*. Ambiguous results can be investigated further by western blot analysis using purified elementary bodies (Jones *et al*., 1997).

Antigen is prepared from heavily infected yolk sac membranes obtained from chicken embryos that have been inoculated in the same manner as those used to isolate the organism from field material. The preparation of the antigen should be carried out in a biosafety cabinet with the appropriate biosecurity precautions to prevent human infection (see Chapter 1.1.2). Chopped and ground membranes are suspended in phosphate buffer, pH 7.6, at the rate of 2 ml per g membrane. After removal of crude debris, the supernatant fluid is centrifuged at 10,000 ***g*** for 1 hour at 4°C, the deposit is resuspended in a small volume of saline, and a smear of this is examined to ensure a high yield of chlamydiae. The suspension is held in a boiling water bath for 20 minutes, or is autoclaved, and sodium azide (0.3%) is added as a preservative. Antigen may also be prepared from cell cultures infected with *C. abortus*. Infected monolayers are suspended in phosphate buffer, pH 7.6, and the cells are disrupted by homogenisation or ultrasonication. Gross debris is removed and subsequent procedures are as for the preparation of antigen from infected yolk sacs. In either case, CF tests with standardised complement and antisera will establish the optimal working dilution for each batch of antigen.

b) Other tests

The serological responses to *C. abortus* and *C. pecorum* can be resolved by indirect micro-immuno-fluorescence, but the procedure is too time-consuming for routine diagnostic purposes. ELISAs developed independently by several research groups have not been adapted for general diagnostic work, partly because of difficulties associated with the use of particulate antigens. However, a novel ELISA that incorporates a stable, solubilised antigen has been used to test experimental and field samples, and has given results that, though lacking species specificity, have a higher sensitivity than the CF test (Anderson *et al*., 1995; Jones *et al*., 1997). Other tests using monoclonal antibody technology in a competitive ELISA (Salti-Montesanto *et al*., 1997) and recombinant antigen technology in indirect ELISAs (Longbottom *et al*., 2002) have been developed and shown to be more sensitive and specific than the CF test in differentiating animals infected with *C. abortus* from those infected with *C. pecorum*. However, these tests are currently mainly used as research tools, and have not been developed commercially. A number of commercially

available serological tests have been evaluated and compared with these 'in-house' tests with variable results (Jones *et al*., 1997; Vretou *et al*., 2007). None of the serological tests that is available can differentiate vaccination titres from those acquired as a result of natural infection (Borel *et al*., 2005).

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

1. Background

a) Rationale and intended use of the product

Currently, two types of vaccine (inactivated and attenuated live vaccines) are available commercially, to be administered intramuscularly or subcutaneously at least 4 weeks before breeding to aid in the prevention of abortion. A multi-component recombinant vaccine against *C. abortus* remains a future goal of chlamydial vaccine research (Longbottom & Livingstone, 2006).

Inactivated vaccines can be prepared from infected yolk sacs or cell cultures (Jones *et al*., 1995) and incorporate whole organisms or fractions of them (Tan *et al*., 1990) using the appropriate biosecurity precautions to prevent human infection (see Chapter 1.1.2). Operator care should be observed in handling commercial inactivated vaccines that incorporate mineral oil-based adjuvants, as self-injection can result in severe local inflammation and tissue necrosis. The commercial live, attenuated vaccine is a chemically induced temperature-sensitive mutant strain of the organism that grows at 35°C but not at 39.5°C, the body temperature of sheep (Rodolakis, 1986). This vaccine is supplied lyophilised and must be reconstituted in diluent immediately before administration. Operator care should be observed in handling and administering this live vaccine, particularly by immunocompromised individuals and pregnant women. Importantly, the live vaccine must not be given to animals being treated with antibiotics, particularly teracyclines.

Both types of vaccine have a role to play in controlling disease, but neither confers absolute protection against challenge or completely reduces the shedding of infective organisms. However, vaccinates exposed to infection do experience significantly lower abortion rates and reduced excretion of chlamydiae for at least two to three lambings after vaccination. It has been claimed that the live vaccine could be an aid to eradication of disease (Nietfeld, 2001).

Vaccine stored under refrigeration (5±3°C) should remain stable for at least 1 year. Before use it should be held at room temperature for 24 hours, and the container should be shaken vigorously immediately before vaccine is withdrawn. No firm data are available, but revaccination is recommended after 1–3 years, according to the exposure risk.

2. Outline of production and minimum requirements for conventional vaccines

~~**1. Seed management**~~

a) Characteristics of the seed

i) Biological characteristics

One or more ovine abortion isolates that consistently grow productively in the chosen substrate are suitable, and an early passage of the seed stock can be established. Alternatively, an isolate that has been adapted to the chicken embryo by multiple passage (>100) can be used. This permits more of the embryo to be used for vaccine production. Although adaptation to the embryo may diminish the isolate's virulence for sheep, there is no evidence that such change reduces its protective efficacy as an inactivated vaccine.

ii) Quality criteria (sterility, purity, freedom from extraneous agents)

Before inoculation of large numbers of embryos or cell cultures, the viability and freedom from contamination of seed stock should be verified. It may be convenient to collect the total harvest in separate manageable lots. In this case, the infectivity of an aliquot of each lot should be separately titrated to ensure that each matches the requirements (see below). Store under refrigerated conditions.

b) Method of manufacture ~~culture~~

i) Procedure

For low passage isolates, the procedures described for the preparation of CF antigen are suitably adapted and amplified for bulk production. Once the final harvest suspension is obtained, an aliquot is removed for titration of its infectivity. The bulk is treated with formalin to a final concentration of 0.4%, and stored until sterility tests confirm complete inactivation.

~~**c) Validation as a vaccine**~~

~~Before inoculation of large numbers of embryos or cell cultures, the viability and freedom from contamination of seed stock should be verified. It may be convenient to collect the total harvest in separate manageable lots. In this case, the infectivity of an aliquot of each lot should be separately titrated to ensure that each matches the requirements (see Section C.2 below). Store under refrigerated conditions.~~

~~**2. Method of manufacture**~~

ii) Requirements for substrates and media

The inactivated harvest is centrifuged and resuspended in phosphate buffered saline containing 0.2% formalin to a volume representing a preinactivation infectivity titre of approximately 10^8 infectious units/ml. Usually, the aqueous suspension is blended with an oil adjuvant, either directly or after precipitation by potassium alum ($AlK[SO_4]_2.12\ H_2O$). A preservative, such as 0.01% thiomersal, may also be added.

~~**3. In-process control**~~

iii) In process controls

The main requirements are to ensure adequate growth of *C. abortus*, avoidance of extraneous infection of the culture substrate, completeness of inactivation and biohazard awareness by process workers.

~~**4. Batch control**~~

iv) Final product batch tests

Each separate batch of manufactured vaccine should be tested for sterility, safety and potency.

~~**a) Sterility**~~

Sterility and purity

Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.9.

~~**b) Safety**~~

Safety

Subcutaneous inoculation into two or more seronegative sheep of twice the standard dose (usually 1.0 ml) of manufactured vaccine should elicit no systemic reaction, but oil-adjuvant vaccines can cause a nonharmful swelling at the inoculation site.

~~**c) Potency**~~

Batch potency

At present, potency is judged by the occurrence of a serological response in previously unvaccinated sheep given 1 ml of vaccine subcutaneously. Blood samples taken before and 28 days after vaccination are compared. Ultimately, potency has to be judged against experimental challenge or field performance, but no *in vitro* correlation of protective efficacy has yet been established.

c) Requirements for authorisation

i) Safety requirements

See Chapter 1.1.8. Principles of veterinary vaccine production.

ii) Efficacy requirements

See Chapter 1.1.8. Principles of veterinary vaccine production

iii) Stability

See Chapter 1.1.8. Principles of veterinary vaccine production

3. Vaccines based on biotechnology

a) Vaccines available and their advantages

No biotechnology-based vaccines are currently in use for this disease.

~~**d) Duration of immunity**~~

~~No firm data are available, but revaccination is recommended after 1–3 years, according to the exposure risk.~~

~~**e) Stability**~~

~~Vaccine stored under refrigeration (5±3°C) should remain stable for at least 1 year. Before use it should be held at room temperature for 24 hours, and the container should be shaken vigorously immediately before vaccine is withdrawn.~~

~~**5. Tests on the final product**~~

~~**a) Safety**~~

~~See Section C.4.b.~~

~~**b) Potency**~~

~~See Section C.4.c.~~

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NB: There are OIE Reference Laboratories for Enzootic abortion of ewes (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).

CHAPTER 2.7.11.

PESTE DES PETITS RUMINANTS

SUMMARY

Peste des petits ruminants (*PPR*), *is an acute contagious disease caused by a Morbillivirus in the family Paramyxoviridae. It affects mainly sheep and goats and occasionally ~~small ruminants living in the~~ wild small ruminants. Based on the fact that PPR has been reported on a few occasions in camels, cattle and buffaloes, those animal species are considered to be susceptible although their potential role in the circulation of PPR virus (PPRV) has not been formally established. PPR occurs in Africa except Southern Africa ~~in countries lying between the Equator and the Sahara~~, in the Arabian Peninsula, throughout most of the Near East and Middle East, and in Central and South-West Asia.*

The clinical disease resembles rinderpest in cattle. It is usually acute and characterised by serous ocular and nasal discharges. PPR is characterised by severe pyrexia, erosive lesions on different mucous membranes and particularly in the mouth, diarrhoea and pneumonia. At necropsy, characteristic zebra markings may occur in the large intestine, but are not a consistent finding. Lesions also occur in the lungs showing congestion or bronchopneumonia when associated with bacterial infection. PPR can also occur in subclinical form.

The disease must be differentiated from rinderpest, bluetongue, foot and mouth disease and other exanthemous conditions.

Identification of the agent: *The collection of specimens at the correct time is important to achieve diagnosis by virus isolation and they should be obtained in the acute phase of the disease when clinical signs are still apparent. The specimens can be swabs of conjunctival discharges, nasal secretions, buccal and rectal mucosae, and unclotted blood.*

Rapid diagnosis is done by immunocapture enzyme-linked immunosorbent assay (*ELISA*)*, counter immunoelectrophoresis and agar gel immunodiffusion. Polymerase chain reaction may also be used.*

Serological tests: *The serological tests that are routinely used are the virus neutralisation and the competitive ELISA.*

Requirements for vaccines and diagnostic biologicals: *In the past, control of PPR was ensured through vaccination with the rinderpest tissue culture vaccine because of the existence of a strong antigenic relationship between PPR and rinderpest viruses. The use of this heterologous vaccine has been abandoned in favour of the live attenuated PPR virus vaccine, which is now widely commercially available.*

A. INTRODUCTION

Peste des petits ruminants (PPR) is an acute viral disease of small ruminants characterised by fever, oculonasal discharges, stomatitis, diarrhoea and pneumonia with foul offensive breath. Infected animals present clinical signs similar to those of rinderpest in cattle, from which it must be differentiated. Because of the respiratory signs, PPR can be confused with contagious caprine pleuropneumonia (CCPP) or pasteurellosis. In many cases, pasteurellosis is a secondary infection of PPR, a consequence of the ~~immunodepression~~ immunosuppression that is induced by the causal agent of PPR, the PPR virus (PPRV). PPRV is transmitted mainly by aerosols between animals living in close contact (Lefevre & Diallo, 1990).

On the basis of its similarities to the viruses of rinderpest, canine distemper and measles, the PPRV has been classified within the genus Morbillivirus in the family Paramyxoviridae (Gibbs *et al.*, 1979). Virus members of this group have six structural proteins: the nucleocapsid protein (Np), which encapsulates the virus genomic RNA, the phosphoprotein (P), which associates with the polymerase (L for large protein) protein, the matrix (M) protein, the

fusion (F) protein and the haemagglutinin (H) protein. The matrix protein, intimately associated with the internal face of the viral envelope, makes a link between the nucleocapsid and the virus external glycoproteins: H and F, which are responsible, respectively, for the attachment and the penetration of the virus into the cell to be infected. PPR was first described in Côte d'Ivoire (Gargadennec & Lalanne, 1942), but it occurs in most African countries ~~south~~ from North Africa to Tanzania ~~of the Sahara and north of the equator~~ (Kwiatek *et al.*, 2011; Lefevre & Diallo, 1990; Swai, *et al.,* 2009), and in nearly all Middle Eastern countries to Turkey (Furley *et al.*, 1987; Lefevre *et al.*, 1991; Ozkul *et al.*, 2002; Perl *et al.*, 1994; Taylor *et al.*, 1990). PPR is also widespread in countries from ~~India~~ central Asia to China, including South and South-~~west~~ East Asia (Banyard et *al.,* 2010; Shaila *et al.*, 1989; Wang *et al.*, 2009).

The natural disease affects mainly goats and sheep ~~but it is usually more severe in goats where it causes heavy losses and is only occasionally severe in sheep~~. It is generally admitted that cattle can only be infected subclinically. However, in poor conditions it might be possible that cattle develop lesions following PPRV infection, clinical signs of which would be ascribed to rinderpest. Indeed, in the 1950s, disease and death were recorded in calves experimentally infected with PPRV-infected tissue (Mornet *et al.*, 1956). The virus that was involved in a natural rinderpest outbreak in cattle, sheep and goats in 1971 in Sudan was, in fact, a PPRV (Kwiatek *et al.*, 2011). Moreover, PPRV was isolated from an outbreak of rinderpest-like disease in buffaloes in India in 1995 (Govindarajan *et al.*, 1997). Not only antibodies to PPRV were detected in camels in Egypt and Ethiopia, but also the virus that was ~~It was also~~ suspected to be involved in the epizootic disease that affected one-humped camels in Ethiopia and Sudan ~~in 1995–1996~~ (Abraham *et al.*, 2005; Ismail *et al.*, 1992; Khalafalla et *al.* 2010; Kwiatek *et al.*, 2011; Roger *et al.*, 2000; 2001). Indeed PPRV antigen and PPRV nucleic acid were detected in some pathological samples collected during that outbreak, but no live virus was isolated. Cases of clinical disease have been reported in wildlife resulting in deaths ~~of gazelles~~ of wild small ruminants ~~in captivity~~ (Abubakar et *al.*, 2011; Bao *et al.*, 2011; Elzein *et al.,* 2004; Furley *et al.,* 1987; Kinne et *al.,* 2010). The American white-tailed deer (*Odocoileus virginianus*) can be infected experimentally (Hamdy & Dardiri, 1976).

The incubation period is 4–6 days, but may range between 3 and 10 days. The clinical disease is acute, with a pyrexia up to 41°C that can last for 3–5 days; the animals become depressed, anorexic and develop a dry muzzle. The serous oculonasal discharges become progressively mucopurulent and, if death does not ensue, persist for around 14 days. Within 4 days of the onset of fever, the gums become hyperaemic, and erosive lesions develop in the oral cavity with excessive salivation. These lesions may become necrotic. A watery blood-stained diarrhoea is common in the later stage. Pneumonia, coughing, pleural rales and abdominal breathing also occur. The morbidity rate can be up to 100% with ~~a mortality rate of up to 100%~~ very high case fatality in severe cases. However, morbidity and mortality may be much lower in ~~this may not exceed 50% during~~ milder outbreaks, and the disease may be overlooked. A tentative diagnosis of PPR ~~is~~ can be made on ~~these~~ clinical signs, but this diagnosis is considered provisional until laboratory confirmation is made ~~is required~~ for differential diagnosis with other diseases with similar signs.

At necropsy, the lesions are very similar to those observed in cattle affected with rinderpest, except prominent crusty scabs along the outer lips and severe interstitial pneumonia frequently occur with PPR. Erosive lesions may extend from the mouth to the reticulo–rumen junction. Characteristic linear haemorrhages or zebra stripes occur in the large intestine, commonly at the caeco–colic junction, but they are not a consistent finding; necrotic or haemorrhagic enteritis is usually present. Lymph nodes are enlarged, the spleen may show necrotic lesions, and there is an apical pneumonia.

There are no known health risks to humans working with PPRV as no report of human infection with the virus exists.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

a) Collection of samples

In live animals, swabs are made of the conjunctival discharges and from the nasal and buccal mucosae. During the very early stage of the disease, whole blood is also collected in anticoagulant for virus isolation, polymerase chain reaction (PCR) and haematology. At necropsy (two to three animals), lymph nodes, especially the mesenteric and bronchial nodes, lungs, spleen and intestinal mucosae should also be collected aseptically, chilled on ice and transported under refrigeration. Fragments of organs collected for histopathology are placed in 10% formalin. At the end of the outbreak, blood can be collected for serological diagnosis.

b) Agar gel immunodiffusion

Agar gel immunodiffusion (AGID) is a very simple and inexpensive test that can be performed in any laboratory and even in the field. Standard PPR viral antigen is prepared from mesenteric or bronchial lymph nodes, spleen or lung material and ground up as 1/3 suspensions in buffered saline. These are centrifuged at 500 ***g*** for 10–20 minutes, and the supernatant fluids are stored in aliquots at –20°C. The cotton material from the cotton bud used to collect eye or nasal swabs is removed using a scalpel and inserted into a 1 ml syringe. With 0.2 ml of phosphate buffered saline (PBS), the sample is extracted by repeatedly expelling and filling the 0.2 ml of PBS into an Eppendorf tube using the syringe plunger. The resulting eye/nasal swab extracted sample, like the tissue ground material prepared above, may be stored at –20°C until used. They may be retained for 1–3 years. Negative control antigen is prepared similarly from normal tissues. Standard antiserum is made by hyperimmunising sheep with 1 ml of PPRV with a titre of 10^4 $TCID_{50}$ (50% tissue culture infective dose) per ml given at weekly intervals for 4 weeks. The animals are bled 5–7 days after the last injection (Durojaiye, 1982). Standard rinderpest rabbit hyperimmune antiserum is also effective in detecting PPR antigen.

i) Dispense 1% agar in normal saline, containing thiomersal (0.4 g/litre) or sodium azide (1.25 g/litre) as a bacteriostatic agent, into Petri dishes (6 ml/5 cm dish).

ii) Wells are punched in the agar following a hexagonal pattern with a central well. The wells are 5 mm in diameter and 5 mm apart.

iii) The central well is filled with positive antiserum, three peripheral wells with positive antigen, and one well with negative antigen. The two remaining peripheral wells are filled with test antigen, such that the test and negative control antigens alternate with the positive control antigens.

iv) Usually, 1–3 precipitin lines will develop between the serum and antigens within 18–24 hours (Durojaiye *et al.*, 1983). These are intensified by washing the agar with 5% glacial acetic acid for 5 minutes (this procedure should be carried out with all apparently negative tests before recording a negative result). Positive reactions show lines of identity with the positive control antigen.

Results are obtained in one day, but the test is not sensitive enough to detect mild forms of PPR due to the low quantity of viral antigen that is excreted.

c) Counter immunoelectrophoresis

Counter immunoelectrophoresis (CIEP) is the most rapid test for viral antigen detection (Majiyagbe *et al.*, 1984). It is carried out on a horizontal surface using a suitable electrophoresis bath, which consists of two compartments connected through a bridge. The apparatus is connected to a high-voltage source. Agar or agarose (1–2%, [w/v]) dissolved in 0.025 M barbitone acetate buffer is dispensed on to microscope slides in 3-ml volumes. From six to nine pairs of wells are punched in the solidified agar. The reagents are the same as those used for the AGID test. The electrophoresis bath is filled with 0.1 M barbitone acetate buffer. The pairs of wells in the agar are filled with the reactants: sera in the anodal wells and antigen in the cathodal wells. The slide is placed on the connecting bridge and the ends are connected to the buffer in the troughs by wetted porous paper. The apparatus is covered, and a current of 10–12 milliamps per slide is applied for 30–60 minutes. The current is switched off and the slides are viewed by intense light: the presence of 1–3 precipitation lines between pairs of wells is a positive reaction. There should be no reactions between wells containing the negative controls.

d) Immunocapture enzyme-linked immunosorbent assay

The immunocapture enzyme-linked immunosorbent assay (ELISA) (Libeau *et al.*, 1994) using ~~three~~ two monoclonal antibodies (MAb) anti-N protein, allows a rapid ~~differential~~ identification of PPRV ~~or rinderpest viruses, and this is of great importance as the two diseases had until recently a similar geographical distribution and may affect the same animal species~~.

i) Microtitre ELISA plates (e.g. ~~high adsorption capacity~~ Nunc ~~Maxisorb~~ Maxisorp) are coated with 100 µl of a capture MAb solution (diluted according to the instructions of the Reference Laboratory providing the kit). ~~This MAb reacts with both rinderpest virus and PPRV.~~

ii) After washing, 50 µl of the sample suspension is added to ~~four~~ two wells, and control wells are filled with buffer.

iii) Immediately, 25 µl of a detection biotinylated MAb for PPR and 25 µl of streptavidin/peroxidase are added to two wells. ~~and 25 µl of a detection MAb for rinderpest and 25 µl of streptavidin/peroxidase are added to the two other wells.~~

iv) The plates are incubated at 37°C for 1 hour with constant agitation.

v) After three vigorous washes, 100 µl of ortho-phenylenediamine (OPD) in hydrogen peroxide is added, and the plates are incubated for 10 more minutes at room temperature.

vi) The reaction is stopped by the addition of 100 µl of 1 N sulphuric acid, and the absorbance is measured at 492 nm on a spectrophotometer/ELISA reader.

The cut-off above which samples are considered to be positive is calculated from the ~~each~~ blank ~~(PPR blank and rinderpest blank)~~ as three times the mean absorbance values.

~~A sandwich ELISA can also be performed: the sample is first allowed to react with the detection MAb and the immunocomplex is then captured by the second MAb adsorbed on to the ELISA plate.~~

The test is very specific and sensitive (it can detect $10^{0.6}$ $TCID_{50}$/well for the PPRV and $10^{2.2}$ $TCID_{50}$ for the rinderpest virus). The results are obtained in 2 hours.

Another immunocapture test, based on the use of a single MAb anti-H, has been described (Saliki *et al.*, 1994).

A sandwich ELISA can also be performed (Singh *et al.*, 2004a): the sample is first allowed to react with the detection MAb and the immunocomplex is then captured by the MAb or polyclonal antibody adsorbed on to the ELISA plate. The assay shows high correlation to the cell infectivity assay ($TCID_{50}$) with a minimum detection limit of 10^3 $TCID_{50}$/ml (Saravanan *et al.*, 2008).

e) Nucleic acid recognition methods

cDNA ^{32}P labelled clones have been used to differentiate PPR and rinderpest (Diallo *et al.*, 1989a), but their use in routine diagnosis is not recommended due to the short half-life of the ^{32}P and the need for special equipment to protect the users.

A reverse transcription PCR (RT-PCR) technique based on the amplification of the Np and F protein genes has been developed for the specific diagnosis of PPR (Couacy-Hymann *et al.*, 2002; Forsyth & Barrett, 1995; Saravanan *et al.*, 2004). This technique is very sensitive compared with other tests and results are obtained in 5 hours, including the RNA extraction. The OIE and FAO[1] Reference Laboratory for PPR in France (see Table given in Part 3 of this *Terrestrial Manual*) can advise on the use of this technique. A multiplex RT-PCR, based on the amplification of fragments of N and M protein genes, has been reported (George *et al.*, 2006). Another format of the N gene-based RT-PCR has also been described (Kumar *et al.*, 2007). Instead of analysing the amplified product – the amplicon – by agarose gel electrophoresis, it is detected on a plate by ELISA through the use of a labelled probe. This new format, RT-PCR-ELISA, is ten times for sensitive that the classical RT-PCR. In recent years, nucleic acid amplification methods for PPR diagnosis have been significantly improved with quantitative RT-PCR (Adombi *et al.*, 2011; Balamurugan *et al.*, 2010; Bao *et al.*, 2008; Kwiatek *et al.*, 2010; Li *et al.*, 2010). The nucleic acid isothermal amplification for PPR diagnosis has also been described (Li *et al.*, 2010).

f) Culture and isolation methods

Even when diagnosis has been carried out by rapid techniques, the virus should always be isolated from field samples in tissue cultures for further studies (Durojaiye *et al.*, 1983; Lefevre & Diallo, 1990).

PPRV may be isolated in primary lamb kidney/lung cells and cell lines (Vero, B95). Unfortunately, PPRV isolation using Vero cells is not always successful and may require multiple blind passages. Recently monkey CV1 expressing the PPRV receptor, the signalling lymphocyte activation molecule (SLAM), has been developed for quick isolation of field viruses from pathological specimens in less than 1 week, without requirement of blind passages (Adombi *et al.*, 2011). ~~or in African green monkey kidney (Vero) cell tissue cultures.~~ Monolayer cultures are inoculated with suspect material (swab material, buffy coat or 10% tissue suspensions) and examined daily for evidence of cytopathic effect (CPE). The CPE produced by PPRV can develop within 5 days and consists of cell rounding and aggregation culminating in syncytia formation in lamb kidney cells and the cell line, CV1 expressing SLAM. In Vero cells, it is sometimes difficult to see the syncytia. If they exist, they are very small. However, in stained, infected Vero cells, small syncytia are always seen. Syncytia are recognised by a circular arrangement of nuclei giving a 'clock face' appearance. Cover-slip cultures may give a CPE earlier than day 5. There are also intracytoplasmic and intranuclear inclusions. Some cells are vacuolated. Similar cellular changes may be seen in stained histopathological sections of infected tissues. After 5–6 days, blind passages should always be carried out as CPE may take time to appear.

1 Food and Agriculture Organization of the United Nations.

g) Other virus detection techniques

Other virus detection techniques have potential benefits, but they are not yet widely used. While virus isolation needs pathological samples to be kept in cold conditions until the start of their processing, it is possible to keep them at ambient temperature in a formalin-fixed solution and later analyse them directly by immunofluorescence (IF) or immunochemical test (Brown *et al.*, 1991; Bundza *et al.*, 1988; Sumption *et al.*, 1998). IF has been used successfully on conjunctival smears and tissues collected at necropsy; the smears are fixed in cold acetone. It has now been demonstrated that unlike the rinderpest virus but like the measles virus, PPRV has haemagglutination capability. This characteristic has been used for specific, rapid and inexpensive diagnosis of PPR infection (Govindarajan *et al.*, 1997; Wosu, 1991).

2. Serological tests

Goats and sheep infected with PPRV develop antibodies that may be demonstrated to support a diagnosis by the antigen-detection tests. Tests that are routinely used are the virus neutralisation (VN) test and the competitive ELISA.

a) Virus neutralisation (the prescribed test for international trade)

This test is sensitive and specific, but it is time-consuming. The standard neutralisation test is carried out in roller-tube cultures of primary lamb kidney cells, or Vero cells when primary cells are not available.

i) Dilute 1 ml of inactivated serum in a twofold dilution series and mix with a stock virus suspension containing approximately 10^3 $TCID_{50}$/ml.

ii) Incubate the virus/serum mixtures either for 1 hour at 37°C or overnight at 4°C.

iii) Inoculate 0.2 ml of the mixture into each of five roller tubes, followed immediately by 1 ml of Vero cell suspension in growth medium at a rate of 2×10^5 cells/ml.

iv) Incubate the sloped tubes for 3 days at 37°C.

v) Discard the tubes showing virus-specific CPE; replace the medium in the remaining tubes with maintenance medium, and roll the tubes for a further 7 days. The virus-challenge dose is acceptable if it falls between $10^{1.8}$ and $10^{2.8}$ $TCID_{50}$/tube. Any detectable antibody at a dilution of 1/8 is considered to be positive.

When ~~Usually,~~ a cross-neutralisation test is carried out with rinderpest virus, ~~and~~ a serum is considered to be positive for PPR when the neutralisation titre is at least twofold higher for PPR than for rinderpest.

Instead of using the roller tubes, the VN test can also be performed in 96-well microtitre plates (Rossiter *et al.*, 1985).

b) Competitive enzyme-linked immunosorbent assay

Competitive ELISA based on the use of MAb anti-nucleoprotein and a recombinant nucleoprotein produced in the baculovirus has been described (Libeau *et al.*, 1995).

i) Coat microtitre plates (e.g. ~~high adsorption capacity~~ Nunc ~~Maxisorb~~ Maxisorp) with 50 µl of a predetermined dilution of N-PPR protein (produced by a recombinant baculovirus) for 1 hour at 37°C with constant agitation.

ii) Wash the plates three times and blot dry.

iii) Distribute 45 µl of blocking buffer (PBS + 0.5% Tween 20 + 0.5 fetal calf serum) to all wells, and then add 5 µl of test sera to test wells (at a final dilution of 1/20) and 5 µl of the different control sera (strong positive, weak positive and negative serum) to control wells.

iv) Add 50 µl of MAb diluted 1/100 in blocking buffer, and incubate at 37°C for 1 hour.

v) Wash the plates three times and blot dry.

vi) Add 50 µl of anti-mouse conjugate diluted 1/1000, and incubate at 37°C for 1 hour.

vii) Wash the plates three times.

viii) Prepare OPD in hydrogen peroxide solution. Add 50 µl of substrate/conjugate mixture to each well. Stop the reaction after 10 minutes with 50 µl of 1 M sulphuric acid.

ix) Read on an ELISA reader at 492 nm.

The absorbance is converted to percentage inhibition (PI) using the formula:

PI = 100 – (absorbance of the test wells/absorbance of the MAb control wells) × 100

Sera showing PI greater than 50% are positive.

~~Two~~ Three other competitive ELISA techniques, based on the use of ~~monoclonal~~ MAb anti-haemagglutinin (H), have also been described (Andersen & McKay, 1994; Saliki *et al.*, 1993; Singh *et al.*, 2004b).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Section under study

~~Sheep and goats that recover from PPR develop an active life long immunity against the disease. As antibodies have been demonstrated 4 years after infection, this immunity is probably life-long (Durojaiye, 1982). Homologous PPR vaccine is available. In 1998, the OIE International Committee endorsed the use of vaccine in countries that have decided to follow the 'OIE pathway' for epidemiological surveillance for rinderpest in order to avoid confusion when serological surveys are performed. There have been two published reports on the preliminary results of the development of recombinant capripox-based PPR vaccine able to protect against both capripox and PPR (Berhe *et al.*, 2003; Diallo *et al.*, 2002). The production of the commercially available attenuated PPRV vaccine is described here.~~

~~1. Seed management~~

~~a) Characteristics of the seed~~

~~The PPRV vaccine Nigeria 75/1 strain is a live vaccine cultured in Vero cells. The original strain of the virus was isolated in Nigeria in 1975 (Taylor & Abegunde, 1979). It has been attenuated by serial passages in Vero cell cultures (Diallo *et al.*, 1989b). The strain provided for vaccine production is the 70th passage in Vero cells (PPRV 75/1 I.K6 BK2 Vero 70). It is stored in freeze-dried form at –20°C and may be obtained from Reference Laboratories (see Table given in Part 3 of this *Terrestrial Manual*). Tests of vaccine activity show that it retained the ability to protect (at a dose of 10^3 $TCID_{50}$) up to the 120th passage in Vero cells, the latest passage tested so far.~~

~~b) Method of culture~~

~~o Cells~~

~~PPR vaccine is produced in Vero cells, which must be free from all bacterial, fungal and viral contamination.~~

~~o Culture medium~~

~~The culture medium consists of minimal essential medium (MEM) supplemented with antibiotics (for example penicillin + streptomycin at final concentrations of 100 IU [International Units]/ml and 100 µg/ml, respectively), and an antifungal agent (nystatin [Mycostatin] at a final concentration of 50 µg/ml). The medium is enriched with 10% fetal calf serum (complete medium) for cell growth. This proportion of serum is reduced to 2% for maintenance medium when the cell monolayer is complete.~~

~~o Primary seed batch of vaccine virus~~

~~This consists of virus in its 70th passage in Vero cells (PPRV 75/1 LK6 BK2 Vero 70). The freeze dried contents of a flask from the seed bank are reconstituted with 2 ml of sterile water (or cell culture medium without serum). This liquid is mixed with Vero cells suspended in complete culture medium to provide at least 0.001 $TCID_{50}$ per cell. Cell culture dishes are filled with this virus/cell mixture (around 2×10^7 Vero cells in a 175 cm^2 dish), and are incubated at 37°C. The cultured cells are examined regularly to detect a CPE. The medium is renewed every 2 days, reducing the proportion of serum to 2% once the cell monolayer is complete. Virus is first harvested when there is 40–50% CPE. This viral suspension is stored at –70°C. Successive harvesting is made every 2 days until the CPE reaches 70–80%, which is the time for final freezing of the culture dishes (in general, at least two further harvestings can be made before final freezing of the culture dishes). All suspensions of virus collected are submitted to two freeze–thaw cycles, then added to form a single batch, which serves as the primary seed batch. This is divided into small volumes in bottles and stored at –70°C. The contents of five flasks are thawed and titrated (minimum titre required: 10^5 $TCID_{50}$/ml). It is best to freeze-dry this seed in order to store it at –20°C. In this case it will be necessary to titrate the freeze-dried virus (five bottles). A batch made up in this way must pass all tests for sterility.~~

~~When preparing seed batches, it is important to avoid infecting the cells with high doses of virus (high multiplicity of infection), as this will lead to accumulation of defective particles in the viral suspension produced, which will diminish the titre of subsequent products. On the other hand, very weak multiplicity of infection (e.g. 0.0001) will prolong the culture time.~~

~~o Preparation of the working seed batch~~

This is done under the same conditions as for the primary seed batch. A large stock of virus is formed, from which the final vaccine will be produced. This batch is distributed into receptacles and stored at –70°C. It must satisfy tests for sterility. Five samples are titrated (minimum titre required: 10^6 $TCID_{50}$/ml).

c) Validation as a vaccine

It is necessary to confirm or rule out the presence of PPRV in the product under test. For this purpose, anti-PPR serum is used to neutralise the virus in cell culture.

o Test procedure

i) Mix the contents of two vaccine bottles with sterile double-distilled water to provide a volume equal to the volume before freeze drying.

ii) Make tenfold dilutions of the reconstituted vaccine in serum free culture medium (0.5 ml of viral suspension + 4.5 ml of medium).

iii) Make two series of mixtures for virus dilutions from each bottle on a 96 well plate as follows:

Series 1: Dilutions of viral suspension:	1	2	3	4
Viral suspension (in µl)	50	50	50	50
Culture medium (in µl)	50	50	50	50

Series 2: Dilutions of viral suspension:	1	2	3	4
Viral suspension (in µl)	50	50	50	50
PPR antiserum (in µl)	50	50	50	50

(*Note: PPR antiserum used for this purpose is prepared in goats and freeze-dried. It is reconstituted with 1 ml of sterile double-distilled water in a dilution of 1/10.*)

iv) Incubate the mixtures at 37°C for 1 hour

v) Add to each well 100 µl of cells suspended in complete culture medium (30,000 cells/well).

vi) Incubate the microplate at 37°C in the presence of CO_2.

vii) Read the plate after 10–15 hours of incubation.

Normally a CPE is present only in the wells containing cells infected with the mixture of virus and culture medium. If it is detected in the wells of Series 2, it will be necessary to identify PPRV by immunofluorescence, using a PPR MAb, or by immunocapture (specific PPR MAb, and the immunocapture test kit are available from the OIE Reference Laboratory for PPR in France [see Table given in Part 3 of this *Terrestrial Manual*]). If this identification confirms the presence of PPRV, the PPR antiserum used must have been too weak, or the batch must be changed. If immunofluorescence or immunocapture is negative, a viral contaminant must be present, and the material under test must be destroyed.

2. Method of manufacture

a) Vaccine production

This operation is performed on a larger scale. Cells can be infected with virus at a multiplicity of infection as before or with high doses, e.g. up to 0.01. Products of the various harvests, after two freeze–thaw cycles, are brought together (to form the final product) and stored at –70°C pending the results of titration and tests for sterility. If these results are satisfactory, the vaccine is freeze-dried.

b) Freeze-drying

The freeze-drying medium (Weybridge medium) is composed of 2.5% (w/v) lactalbumin, 5% (w/v) sucrose and 1% (w/v) sodium glutamate, pH 7.2.

This medium is added to an equal volume of viral suspension for freeze drying (which may have been diluted beforehand to provide the desired number of vaccine doses per bottle). The resulting mixture is kept cool, homogenised, then distributed into bottles and freeze-dried. At the end of a freeze-drying cycle, the probe is adjusted and kept at 35°C for 4 hours. Once this operation has been completed, the bottles are capped under vacuum. Randomly selected samples (e.g. 5%) of this final batch are submitted to tests for innocuousness, efficacy and sterility, and residual moisture is estimated by the Karl Fisher method (optimum ≤3.5%). If the tests give unsatisfactory results, the entire batch is destroyed.

3. In-process control

Cells used in cultures must be checked for normal appearance and shown to be free from contaminating viruses, especially bovine viral diarrhoea virus. A virus titration must be undertaken on the seed lot: using MEM (serum-

free) medium, a series of tenfold dilutions is made (0.5 ml virus + 4.5 ml diluent) down to 10^{-6} of the product to be titrated. Vero cells from one flask are trypsinised and suspended in complete culture medium at 300,000/ml. They are distributed on a 96 well plate (30,000 cells per well, equivalent to 100 µl of cell suspension). Then, 100 µl of virus diluted tenfold is added to the cells (dilutions ranging from 10^{-2} to 10^{-6}). One row of wells serves as a control for uninfected cells to which virus-free culture medium (100 µl) is added. The plate is incubated at 37°C in the presence of CO_2. The plates are read (by examining for CPE) 10–15 days after infection.

Virus titre is determined by the Spearman–Kärber method. The minimum titre per dose is $10^{2.5}$.

4. Batch control

a) Identity

The contents of one container from each filling lot must be checked for identity by culture after neutralisation with specific antiserum.

b) Sterility

This consists of testing for viral, bacterial or fungal contaminants. It is done on cells and sera before their use in vaccine production, and on the seed stock and the vaccine before and after freeze-drying. Any product that fails this test for sterility is destroyed.

Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.9.

c) Safety

This test is done in rodents in order to detect any nonspecific toxicity associated with the product. The test requires reconstituted vaccine in solvent (mixed contents of five bottles), six guinea pigs, each weighing 200–250 g; ten unweaned mice (17–22 g, Swiss line or similar).

Vaccine, 0.5 ml, is injected intramuscularly into a hind limb of two guinea pigs, 0.5 ml into the peritoneal cavity of two guinea pigs, and 0.1 ml into the peritoneal cavity of six mice. Two guinea pigs and four mice are kept as uninoculated controls. The animals are observed for 3 weeks. If one guinea pig or two mice die, the test must be repeated. Dead animals undergo post-mortem examination to ascertain the cause of death. At the end of 3 weeks of observation, all animals are killed for post mortem examination. All the results are recorded. The vaccine is considered to be satisfactory if, during the first or second test, at least 80% of animals remain in good health during the period of observation, and no significant post-mortem lesion is found.

d) Potency and efficacy in small ruminants

This test requires the following: vaccine reconstituted with normal saline (the mixed contents of five bottles) to provide 100 doses and 0.1 dose/ml; six goats and six sheep, all approximately 1 year old and free from antibodies to rinderpest or PPR; sterile syringes and needles; and pathogenic PPRV previously titrated in goats, diluted with sterile normal saline to provide 10^3 of the 50% lethal dose for goats (LD_{50}).

Vaccinate two goats and two sheep subcutaneously with 100 doses per animal; vaccinate two goats and two sheep subcutaneously with 0.1 dose per animal; keep the remaining animals as in-contact controls. The animals are observed and temperature measurements are recorded daily for 3 weeks. At the end of this period, blood is taken from all animals for the preparation of sera. All animals are challenged by subcutaneous injection of a 1 ml suspension of pathogenic PPRV (10^3 LD_{50} per animal). The animals are observed and their body temperature measurements are recorded daily for 2 weeks.

The vaccine is considered to be satisfactory if all vaccinated animals resist the challenge infection, while at least half of the in-contact controls develop signs of PPR. The serum neutralisation test must be positive for PPR antibody (in serum diluted at least 1/10) in vaccinated animals only in samples taken 3 weeks after vaccination. If any of the controls are also positive, the experiment must be repeated using another batch of pathogenic PPRV. The batch of vaccine is destroyed if vaccinated animals react to the virulent challenge.

o Titration of neutralising PPR antibody

This test requires the following: cell suspensions at 600,000/ml; 96-well cell culture plates; sera to be titrated (inactivated by heating to 56°C for 30 minutes); complete cell culture medium; PPRV diluted to give 1000, 100, 10 and 1 $TCID_{50}$/ml.

Dilute the sera at 1/5, then make a twofold serial dilutions in cell culture medium. Mix 100 µl of virus at 1000 $TCID_{50}$/ml (to give 100 $TCID_{50}$ in each well) and 100 µl of a given dilution of serum (using six wells per

dilution) in the wells of the cell culture plate. Arrange a series of control wells for virus and uninfected cells as follows: six wells with 100 $TCID_{50}$ (100 µl) per well; six wells with 10 $TCID_{50}$ (100 µl) per well; six wells with 1 $TCID_{50}$ (100 µl) per well; six wells with 0.1 $TCID_{50}$ (100 µl) per well; and six wells with 200 µl of virus free culture (control cells) per well.

Make the wells containing the virus controls up to 100 µl with complete culture medium, and incubate the plates for 1 hour at 37°C. Add 50 µl of cell suspension to each well. Incubate the plates at 37°C in the presence of CO_2. Read the plates after 1 and 2 weeks of incubation. The results should be as follows: 100% CPE in virus control wells of 100 and 10 $TCID_{50}$, 50% CPE for the 1 $TCID_{50}$ dilution, no CPE for the 0.1 $TCID_{50}$ dilution, no CPE in wells where the virus had been neutralised by serum during the test, and CPE in wells where the virus had not been neutralised by serum during the test.

e) Duration of immunity

Duration of immunity is at least 3 years.

f) Stability

Freeze-dried vaccine can be kept for at least 2 years at 2–8°C (although storage at –20°C is better), provided it is stored under vacuum and protected from light. Recently, it has been demonstrated that this vaccine, suspended in medium containing trehalose and submitted to the ultra-rapid method of dehydration, can resist at 45°C for a period of 14 days with minimal loss of potency (Worrwall *et al.*, 2001).

5. Tests on the final product

a) Safety

See Section C.4.c.

b) Potency

See Section C.4.d.

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* *

NB: There are OIE Reference Laboratories for Peste des petits ruminants (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).

ADVICE FOR MEMBER COMMENTS

Chapter Number and Title: 2.8.2. Atrophic rhinitis of swine

Country making the comments

Date

It would be appreciated if the following guidance notes are followed in making a reply:

1. *Comments may be general or specific, but specific comments are usually more valuable. General comments should be such that some conclusion and action can be taken in response to them. For example, it is of little help to make statements such as "This test is no longer used in our laboratory", without indicating the reasons and what test is used instead.*

2. *Specific comments should be identified, by indicating the line number in the text, to facilitate the making of editorial changes.*

3. *The pointing out of typing or technical errors is welcome, but the correct word or figure should be indicated. For example, it is of little help to say that 0.8 M is too high, if the preferred value is not indicated.*

4. *Bear in mind that the introductory chapters are not intended to be exhaustive, and indeed none of the chapters can give a completely comprehensive cover of the subject, otherwise the* Terrestrial Manual *would be too long. However, assistance in indicating priorities is always helpful.*

5. *The* Terrestrial Manual *is intended for world-wide use. The chapters need to reflect the development of new technology, but the old established methods, usually requiring less sophisticated apparatus, have to be retained. New technology should not be described in detail until it has gained wide acceptance as a reliable method.*

General Comments

Specific Comments (*add continuation sheets if required*)
line:

CHAPTER 2.8.2.

ATROPHIC RHINITIS OF SWINE

SUMMARY

Definition of the disease: *Atrophic rhinitis is an infectious disease of swine characterised by serous to mucopurulent nasal discharge, shortening or twisting of the snout, atrophy of the turbinate (conchal) bones and reduced productivity. It may occur enzootically or more sporadically, depending on a variety of factors including herd immunity. The most severe progressive form is caused by infection with toxigenic strains of* Pasteurella multocida *alone or in combination with* Bordetella bronchiseptica*. Infections with* B. bronchiseptica *alone can cause a mild to moderate form with nonprogressive turbinate bone atrophy. Turbinate atrophy may only be obvious at slaughter or may be detected in the live animal by use of radiography or tomography. Environmental and management factors also contribute to the severity and incidence of this disease. A large proportion of apparently normal pig herds may be infected with* B. bronchiseptica *or nontoxigenic* P. multocida and show a mild degree or low prevalence of turbinate atrophy.

Identification of the agents: *The diagnosis of atrophic rhinitis depends on clinical and post-mortem observations in affected swine assisted by the recovery and characterisation of* P. multocida *and* B. bronchiseptica*. The isolation of both organisms is often complicated by the more profuse growth of other bacteria. Isolation rates are improved by preservation of the nasal or tonsillar swab at 4–8°C in a non-nutritive transport medium and by using a selective culture medium.*

Pasteurella multocida *and* B. bronchiseptica *can be identified by traditional biochemical tests.* Pasteurella multocida *isolates may be further characterised by their capsular and somatic antigens. Capsular type D is most prevalent in many areas of the world, but in some regions type A predominates. Capsular antigens may be distinguished serologically by indirect haemagglutination or immunofluorescence, chemically by flocculation in acriflavine, or by susceptibility to hyaluronidase. Somatic antigen types can be distinguished by a gel diffusion precipitation test, with type 3 found most frequently in swine. Toxigenicity of* P. multocida *isolates can be demonstrated by testing for cytotoxicity in cultured cells. A commercially available toxin-specific enzyme-linked immunosorbent assay (ELISA) is now widely used in some areas of the world to differentiate toxigenic from nontoxigenic isolates. In addition, it is suitable for detection of toxin production by bacteria from primary culture plates without the need for prior isolation and identification of individual colonies.*

Recently developed assays based on the use of DNA probes or polymerase chain reaction (PCR) provide rapid, sensitive and highly specific detection of B. bronchiseptica *and both toxigenic and nontoxigenic* P. multocida *for those laboratories with the capability to perform them. A multiplex PCR useful for capsular typing of* P. multocida *has also been described.*

Serological tests: *Detection of antibodies to* P. multocida *and* B. bronchiseptica *is of little value as nontoxigenic strains of* P. multocida *share cross-reactive antigens with toxigenic strains and* B. bronchiseptica *can be isolated from many swine herds. A test based on detection of antibodies to the* P. multocida *toxin is commercially available but its usefulness is limited as not all infected swine develop such antibodies. Widespread vaccination with* P. multocida *toxoid induces antibodies of vaccinal origin, complicating interpretation of results.*

Requirements for vaccines and diagnostic biologicals: *Several vaccines are available commercially that contain bacterins of* B. bronchiseptica *and a mixture of toxigenic and/or nontoxigenic strains of* P. multocida*, or a toxoid derived from* P. multocida *or from a recombinant* Escherichia coli.

A. INTRODUCTION

Definition of the disease: Atrophic rhinitis is an infectious disease of swine characterised by stunted development or deformation of the nasal turbinate (conchal bone) and septum. ~~The initial clinical signs are sneezing, snuffling and eye discharge with resultant dark tear staining and subsequent nasal discharge, which can vary from serous to mucopurulent; in some cases pigs may show epistaxis. Atrophy of the nasal turbinate (conchal bone) and septal deviation may lead to shortening or twisting of the snout and, in severe cases, difficulty in eating.~~

Causal pathogens: Two forms of atrophic rhinitis have been recognised, depending on the causal agent(s) (De Jong, 2006):

a) A severe progressive form caused by toxigenic isolates of *Pasteurella multocida,* most commonly capsular types D or A, alone or in combination with *Bordetella bronchiseptica.*

b) A less severe, nonprogressive form with mild to moderate turbinate atrophy, often without significant snout changes, caused by *B. bronchiseptica*.

Description of the disease: Initial clinical signs include sneezing, snuffling and eye discharge with resultant dark tear-staining and subsequent nasal discharge, which can vary from serous to mucopurulent; in some cases pigs may show epistaxis. Atrophy of the nasal turbinate and septal deviation may lead to shortening or twisting of the snout and, in severe cases, difficulty in eating. Increased severity is associated with overstocking and poor management, housing and environmental conditions. Reduced productivity is generally associated with moderate to severe atrophic rhinitis, although the precise relationship between infection with these bacteria and reduced weight gains has not been thoroughly elucidated. ~~*Bordetella bronchiseptica* and toxigenic *P. multocida* are commonly found in many domesticated and wild animal species that could potentially transmit the bacteria to swine herds.~~

Bordetella bronchiseptica or toxigenic *P. multocida* may be present in a herd without clinical evidence of disease, especially when other respiratory pathogens are absent and environmental and management conditions are optimal. Such carrier herds pose a risk of transmitting these agents to other herds in which progression to severe disease may occur. *Bordetella bronchiseptica* and toxigenic *P. multocida* are commonly found in many domesticated and wild animal species that could potentially transmit the bacteria to swine herds.

~~B. DIAGNOSTIC TECHNIQUES~~

Zoonotic risk and biosafety requirements: Infection of humans by *B. bronchiseptica* is rare and occurs most often in immunocompromised persons exposed to infected or vaccinated pets; transmission from swine to humans has not been reported. *Pasteurella multocida* is frequently isolated from healthy human carriers working in swine production sites or living nearby and has additionally been associated with chronic or acute respiratory disease in such individuals (Donnio *et al.,* 1999; Marois *et al.,* 2009). Transmission occurs primarily through bites or scratches and wound contamination from infected material, but may also result from inhalation of aerosols. Appropriate precautions should be observed by persons having contact with swine infected with *P. multocida*, particularly those who may be immunocompromised. Containment level 2 practices are recommended for handling clinical specimens and cultures of *B. bronchiseptica* and *P. multocida*.

Differential diagnosis: Porcine cytomegalovirus also causes rhinitis in young pigs but does not progress to turbinate atrophy or nasal distortion. Asymmetric bone development resulting from habitual biting or chewing of stalls or drinkers may lead to a noticeable misalignment of the jaw in some pigs. Careful inspection can distinguish this condition from shortness or deviation of the snout characteristic of atrophic rhinitis.

B. DIAGNOSTIC TECHNIQUES

The diagnosis of atrophic rhinitis depends on clinical, pathological and microbiological investigations, with the latter being particularly important for herds infected subclinically. It is generally accepted that a herd in which toxigenic *P. multocida* is present be defined as affected with progressive atrophic rhinitis, whether or not clinical signs of the disease are evident (Pedersen *et al.,* 1988). Control in many countries has, therefore, centred on detection of infection, even in ~~asymptomatic~~ subclinically infected animals considered to be potential carriers.

1. Pathological diagnostic criteria

Turbinate atrophy may only be seen at slaughter when snout sections at the level of the first/second premolar tooth are examined. Subjective assessment of turbinate atrophy is convenient and often useful for monitoring herds (De Jong, 2006), but objective scales of measurement (Gatlin *et al.,* 1996) are best suited for studies

requiring data analysis. Radiography (Done, 1976) and tomography (Magyar *et al.,* 2003) can provide objective observations from live animals; tomography reveals not only severe lesions but more subtle changes that may not be apparent by radiography. However, these techniques are of limited use due to the equipment and expertise required. Diagnosis is assisted by detection of characteristic histopathological features including fibrous replacement of the bony plates of the ventral conchae with varying degrees of inflammatory and reparative changes.

2. Identification of the agents

a) *In vitro* culture

As *P. multocida* preferentially colonises the tonsil, tonsillar swabs or biopsies will provide the highest isolation rates (Ackermann *et al.,* 1994). Nasal swabs are preferred for isolation of *B. bronchiseptica*. When sampling the tonsil is not practical, nasal swabs suffice for isolation of both organisms. Swabs with flexible shafts should be used; sample collection in young pigs is facilitated by the use of mini-tipped swabs. A single swab is used to sample both sides of the nasal cavity and should then be placed in a non-nutritive transport medium (e.g. phosphate buffered saline) and kept at 4–8°C during transit to avoid overgrowth by other faster-growing bacteria. Transit time should not exceed 24 hours.

Although both *P. multocida* and *B. bronchiseptica* grow readily on blood agar, a selective medium is preferred as overgrowth of other bacteria that are present in higher numbers often interferes with their detection. An additional difficulty related to *B. bronchiseptica* is that this organism grows more slowly than most other bacteria present in clinical samples. Various formulations of media containing antibiotics have been used for isolation of *P. multocida,* but comparison of studies in the literature indicates that the highest isolation rates are obtained with modified Knight medium (bovine blood agar containing 5 µg/ml clindamycin, 0.75 µg/ml gentamicin) (Lariviere *et al*., 1993) or KPMD (bovine blood agar containing 3.75 U/ml bacitracin, 5 µg/ml clindamycin, 0.75 µg/ml gentamicin, and 2.25 µg/ml amphotericin B) (Ackermann *et al*., 1994). MacConkey agar with 1% glucose and 20 µg/ml furaltadone is used by many laboratories for selective growth of *B. bronchiseptica* from nasal swabs, but a modified Smith-Baskerville medium (a peptone agar formulation containing 20 µg/ml penicillin, 20 µg/ml furaltadone, and 0.5 µg/ml gentamicin) appears superior, especially when the number of *B. bronchiseptica* present is low (Lariviere *et al*., 1993; Smith & Baskerville, 1979). A further improvement in isolation rate was reported using blood agar containing 40 µg/ml cephalexin (Lariviere *et al*., 1993). A selective blood agar medium for simultaneous isolation of both *P. multocida* and *B. bronchiseptica*, containing 5 mg/litre clindamycin-HCl, 0.75 mg/litre gentamicin sulphate, 2.5 mg/litre K-tellurite, 5 mg/litre amphotericin-B and 15 mg/litre bacitracin, has also been described (De Jong & Borst, 1985). However, it should be noted that K-tellurite has sometimes been found to be inhibitory to the growth of type D *P. multocida* (Lariviere *et al*., 1993).

b) Biochemical characteristics

P. multocida is a Gram-negative, bipolar, pleomorphic rod and forms nonhaemolytic, greyish colonies on blood agar with a characteristic, 'sweetish' odour ~~(29)~~. It fails to grow on MacConkey agar but yields positive oxidase and catalase reactions and produces indole.

B. bronchiseptica is also a Gram-negative rod, forming convex colonies 1–2 mm in size, usually haemolytic, on blood agar or Bordet-Gengou medium after 48 hours of growth ~~(30)~~. It is nonfermentative, but positive for oxidase, catalase, citrate and urea and grows in 6.5% NaCl.

Agglutination tests using specific antisera have been described for confirming the identity of presumptive *B. bronchiseptica* isolates but appropriate sera are not widely available for use.

- **Capsular typing of *P. multocida***

Capsular typing of *P. multocida* is useful for epidemiological purposes. Serotyping by indirect haemagglutination has traditionally been used (Carter, 1955) but only a few laboratories throughout the world make and maintain the antisera required. However, simpler chemical methods can usually distinguish most swine isolates. Those producing a type D capsule form a heavy flocculate in 1/1000 aqueous acriflavine (Carter & Subronto, 1973), while capsular type A strains can be identified by inhibition of growth in the presence of hyaluronidase (Carter & Rundell, 1975). A small proportion of swine isolates are noncapsulated.

- **Acriflavine test procedure for capsular type D *P. multocida***

i) Inoculate a tube containing 3 ml of brain–heart infusion broth, using a freshly grown bovine blood agar culture, for each *P. multocida* isolate to be tested. Include a known type D strain and a known type A strain as positive and negative controls.

ii) Incubate inoculated tubes at 37°C for 18–24 hours.

iii) Pellet bacteria by centrifugation and remove 2.5 ml of the supernatant.

iv) Add 0.5 ml of a 1/1000 aqueous solution of acriflavine neutral. Acriflavine solution should be freshly prepared each week and stored at 4°C, protected from light.

v) Mix to resuspend the bacterial pellet and incubate the tube at room temperature, without shaking.

vi) Observe at 5 minutes for the presence of a heavy flocculent precipitate.

- **Hyaluronidase test procedure for capsular type A *P. multocida***

i) Prepare fresh bovine blood agar cultures of the isolates to be tested. Include a known type A strain and a known type D strain as positive and negative controls.

ii) Inoculate each strain to be examined on a separate trypticase soy blood agar plate with 6% bovine blood by streaking several parallel lines of growth, approximately 3–5 mm apart, across the diameter of the plate. For maximum production of hyaluronic acid it is important that the plates be fresh and not dehydrated.

iii) Heavily streak a hyaluronidase-producing strain of *Staphylococcus aureus* at right angles to the lines of *P. multocida* growth.

iv) Incubate the plates at 37°C, in a humidified atmosphere, and observe periodically for up to 24 hours. Type A strains will exhibit a marked inhibition of growth in the region adjacent to the growth lines of *S. aureus*.

- **Somatic antigen typing of *P. multocida***

Differences in the cell wall lipopolysaccharide among *P. multocida* strains provide the basis for somatic antigen typing. Sixteen types can be distinguished by a gel diffusion precipitation test (Heddleston *et al*., 1972), with type 3 found most frequently in swine. Although the required antisera are not widely available, many reference laboratories and some diagnostic laboratories offer somatic antigen typing.

- **Detection of the *P. multocida* toxin**

Diagnosis of progressive atrophic rhinitis depends upon characterisation of *P. multocida* isolates as toxigenic. The heat-labile toxin of *P. multocida* produces dermonecrosis in guinea pigs and is lethal in mice following intraperitoneal injection. Toxigenicity can also be demonstrated *in vitro* by testing for cytopathic effects on monolayers of embryonic bovine lung (EBL) cells (Rutter & Luther, 1984), African green monkey kidney (Vero) cells (Pennings & Storm, 1984) or bovine turbinate cells (Eamens *et al*., 1988). The bacteria are grown in brain–heart infusion broth incubated at 37°C for 24 hours and then pelleted by centrifugation. The supernatant is sterilised by filtration and titrated in monolayer cultures prepared in microtitre plates. Following incubation at 37°C for 2–3 days, the monolayers are stained with crystal violet and examined microscopically to detect cytopathic effects. A rapid cell culture test, in which the suspect colonies are grown on an agar overlay of EBL cells (Chanter *et al*., 1986), permits more efficient analysis of large numbers of isolates.

An enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies can detect the toxin in mixtures of bacteria recovered from primary isolation media (Foged, 1992). This is an important advantage as swine may be colonised simultaneously with a mixture of toxigenic and nontoxigenic strains (Ackermann *et al*., 1994; De Jong, 2006). Cell culture methods would require every colony of *P. multocida* in the sample to be tested, which is clearly impractical, to achieve the same level of sensitivity as the ELISA.

This ELISA is commercially available throughout Europe and in a few other selected regions of the world[1] (though not in the United States of America) and has been widely adopted in many areas as the preferred test for identification of carriers and for control of progressive atrophic rhinitis. Though highly specific, a positive result without previous history of disease or suspicious signs should be thoroughly investigated to recover toxigenic isolates from the animals sampled.

c) Molecular ~~Nucleic acid recognition~~ methods

Colony morphology and biochemical testing remain the basis for identification of toxigenic *P. multocida* and *B. bronchiseptica* in many laboratories. However, a number of recently described assays based on the use of DNA probes (Kamps *et al*., 1990; Register *et al*., 1998) or polymerase chain reaction (PCR) (Kamp *et al*., 1996; Lichtensteiger *et al*., 1996; Nagai *et al*., 1994; Register & DeJong, 2006) for detection of toxigenic *P. multocida* and/or *B. bronchiseptica* from swine show promise as faster, more specific, and more sensitive diagnostic tools. Diagnostic laboratories are increasingly using PCR for identification of these agents as the

1 For availability/purchase information contact DakoCytomation Denmark A/S, Produktionsvej 42, DK-2600 Gloistrup, Denmark; http://www.dakocytomation.com.

equipment and expertise become more readily available. Proper in-house validation with known controls as well as standardised, ongoing quality control measures are essential (see Chapter 1.1.4/5 Principles and methods of validation of diagnostic assays for infectious diseases).

A multiplex PCR assay for capsular typing of *P. multocida* (Townsend *et al*., 2001) ~~that~~ appears to provide more reliable results than phenotypic methods ~~has recently been described (37)~~ and is frequently ~~may be of~~ used in suitably equipped diagnostic laboratories.

Various DNA fingerprinting techniques, including restriction endonuclease analysis (REA), ribotyping, pulsed-field gel electrophoresis, and PCR-based methods have been evaluated by numerous groups for the purpose of differentiating *P. multocida* isolates. Few direct comparisons between methods have been carried out using strains from pigs with atrophic rhinitis, but REA currently appears to be the method of choice for epidemiologic investigations as it provides a high level of discrimination without the need for specialized equipment or reagents (Djordjevic *et al*., 1998; Gardner *et al*., 1994; Harel *et al*., 1990)

3. Serological tests

At present there are no satisfactory serological tests that can be relied on to detect those animals infected with toxigenic *P. multocida* and capable of developing or spreading the disease. Detection of antibodies to *P. multocida* is not helpful, as nontoxigenic strains share many cross-reacting antigens with toxigenic strains. An ELISA for detection of antibodies to the *P. multocida* toxin has been described (Foged, 1992) that is commercially available in Europe and a few other areas of the world (see footnote 1). However, many animals infected with toxigenic *P. multocida* fail to produce antibodies to the toxin, and widespread use of toxoid-containing vaccines limits the diagnostic value of this ELISA to herds with no history of vaccination or to detection of vaccine response in vaccinated herds.

Infection with *B. bronchiseptica* can be detected serologically by agglutination testing with formalin-treated bacteria or with a more sensitive ELISA (Venier *et al*., 1984). Unless monitoring the status of a negative herd, *B. bronchiseptica* serology may be of little value as the organism is present in many apparently healthy pig herds.

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

1. Background

There are several commercially available vaccines that contain whole-cell bacterins of *B. bronchiseptica* combined with ~~and~~ a ~~mixture of~~ toxigenic ~~and nontoxigenic~~ *P. multocida* bacterin and/or a *P. multocida* toxoid. The toxigenic *P. multocida* bacterin component is most often capsular type D but some vaccines additionally include a type A strain, which may be toxigenic or nontoxigenic. Live, attenuated *B. bronchiseptica* vaccines are also available. Vaccines containing only *B. bronchiseptica* are not suitable for control of progressive atrophic rhinitis, but may be of benefit in herds with the nonprogressive form. *Pasteurella multocida* and *B. bronchiseptica* vaccines appear to reduce the level of colonisation by these bacteria, but do not eliminate them or prevent infection. Most commercially available vaccines contain either an oil adjuvant or aluminium hydroxide gel.

The *P. multocida* toxin is the single most important protective antigen with respect to progressive atrophic rhinitis. Vaccines based on a *P. multocida* toxoid offer specific protection against the action of the toxin, which, by itself, can be used to reproduce all of the major signs of this disease (see ref. 14 for review). The level of toxin produced by *P. multocida* is relatively low and the toxin-specific antibody response induced by bacterin-only vaccines may not be optimal. Purified toxoid (inactivated by formaldehyde) is more immunogenic than crude toxoid and the immunogenicity of the inactivated form is not affected by mixture with a *B. bronchiseptica* bacterin. However, the difficulty and expense of large-scale purification from cultures of *P. multocida* prevent routine incorporation of native, chemically inactivated ~~purified~~ toxoid into vaccines. Recombinant vaccines containing subunit toxin proteins or genetically detoxified derivatives of the full-length toxin are highly efficacious and less costly to produce (Hsuan *et al.,* 2009; Pejsak *et al.,* 1994). ~~Field studies have shown that a recombinant *P. multocida* toxin derivative, missing a segment of the amino-terminal portion of the protein, is non-toxic but immunogenic and has superior efficacy in swine (2, 27). More recently, full-length recombinant toxoid engineered to contain two amino acid substitutions that eliminate toxigenicity was also found to be highly efficacious (36).~~ A DNA vaccine encoding a full-length but enzymatically inactive toxoid was shown to be highly immunogenic in pigs but has so far not been evaluated for efficacy against challenge (Register *et al*., 2007).

Bordetella bronchiseptica produces a variety of toxins and adhesins that are potential virulence factors in swine. Only one, the outer membrane protein pertactin, has been shown to protect against disease in pigs (Kobisch & Novotny, 1990). Despite this fact, a dermonecrotic toxin produced by *B. bronchiseptica*, unique from the toxin produced by *P. multocida*, has traditionally been regarded as the primary virulence factor and protective

immunogen in swine (De Jong, 2006). Several studies strongly implicate the toxin as a virulence factor and it undoubtedly plays a role in pathogenesis and, perhaps, in protection. However, the role of pertactin and several additional virulence factors in protective immunity is most likely equal to, or perhaps exceeds, that of the toxin.

Bordetella bronchiseptica is subject to phenotypic variation under certain growth conditions (e.g. temperatures below 37°C or the presence of chemical modulators such as $MgSO_4$ or nicotinic acid), in which production of most virulence factors is reversibly turned off. Spontaneously occurring mutants, permanently unable to produce most virulence factors, also arise with a low frequency during culture. Careful attention to colony morphology, on an area of the plate with well-separated colonies, is essential to retain cultures in the phase I (also known as Bvg^+), or virulent, mode. Phase I colonies are small (1–2 mm in diameter), domed, and haemolytic on blood agar. Loss of haemolysis and the appearance of larger, flat colonies indicate conversion to the avirulent form. Whenever possible, cultures should be propagated using single haemolytic colonies to minimise the slow accumulation of avirulent clones within the culture.

~~Precise details of standards for the production of effective commercial vaccines are not available, but they are known to contain 10^{10} cells of formalin killed *B. bronchiseptica* and 10 µg of *P. multocida* toxoid per dose. It is also clear that purified toxoid (inactivated by formaldehyde) is more immunogenic than crude toxoid, and that the immunogenicity of the inactivated form is not affected by mixture with a *B. bronchiseptica* bacterin. The truncated, recombinant *P. multocida* toxin derivative has been inactivated by deletion of a portion of the gene that does not appear to compromise protective immunogenicity. All commercially available vaccines contain either an oil adjuvant or aluminium hydroxide gel.~~

~~*Bordetella bronchiseptica* used for vaccine production should be a phase I virulent culture and the *P. multocida* isolates used should be toxigenic. Seeds of *B. bronchiseptica* and toxigenic *P. multocida* of established identity and passage history should be stored by conventional means. A defined number of passages should be used to give the production culture. *Bordetella bronchiseptica* should be inactivated by formaldehyde. As the toxin of *P. multocida* has an intracellular location and is released on cell lysis during the stationary phase, the culture supernatant should be harvested approximately 48 hours after the end of the exponential phase of growth.~~

~~1. Seed management~~

2. Outline of production and minimum requirements for conventional vaccines

a) Characteristics of the seed

i) *Biological characteristics*

The seed-lot system should be employed for the bacterial strains used to prepare whole-cell bacterins, as well as for the strains from which purified antigens are derived.

In the case of whole-cell bacterins, the origin and history of both the *P. multocida* and *B. bronchiseptica* strains should be described and the full characterisation of the master seeds should be laid down in a master seed batch protocol. *Bordetella bronchiseptica* used for vaccine production should be a phase I virulent culture and the *P. multocida* isolates used should be toxigenic.

Working seeds used for vaccine production should be derived from the master seed and checked for all relevant properties as described in the master seed batch protocol.

ii) *Quality criteria*

Both the master seed and the working seed must be pure cultures, free from bacterial, mycotic, mycoplasmal and viral contamination.

Identity of the bacterial species and the production of relevant antigens should be confirmed.

b) Method of manufacture

i) *Procedure*

Precise details of standards for the production of effective commercial vaccines are not available, but they are known to contain 10^{10} cells of formalin-killed *B. bronchiseptica* and 10 µg of *P. multocida* toxoid per dose. *Bordetella bronchiseptica* should be confirmed to be a phase I culture and, for *P. multocida*, it should be confirmed that the culture contains sufficient levels of toxin. A defined number of passages should be used to give the production culture. *Bordetella bronchiseptica* cells, and either *P. multocida* cells and/or toxin, are inactivated, detoxified and formulated with an adjuvant. As the toxin of *P. multocida* has an intracellular location and is released on cell lysis during the stationary

phase, the culture supernatant should be harvested approximately 48 hours after the end of the exponential phase of growth.

ii) *Requirements for media*

~~**b) Method of culture**~~

All bacterial strains should be propagated in media that support efficient growth and allow optimal expression of the antigens that are relevant for the induction of protective antibodies ~~cultivated in suitable media that efficiently support growth and the expression of relevant antigens~~.

iii) *In-process controls*

Seed and production cultures are inoculated on blood agar plates and incubated. No nonspecific colonies should grow on these plates.

Cultures are inactivated with formaldehyde. Tests are performed to check the effectiveness of the inactivation process and to test for residual formaldehyde.

Quantification of antigens is carried out by performing a total cell count using a bacterial counting chamber for enumerating whole cells or an antigenic mass determination for defined antigens, e.g. *P. multocida* toxin, by quantitative enzyme immunoassay.

iv) *Final product batch tests*

Every batch of vaccine should be tested for sterility according to standard methods (see Chapter 1.1.9 Tests for sterility and freedom from contamination of biological materials) described in the European Pharmacopoeia or the United States Code of Federal Regulations.

Every batch of vaccine should be tested for safety in the target animal, by giving a double dose by the recommended route of vaccination, and a second, single dose 2 weeks later. No abnormal local or systemic reactions should occur. When a preservative is used, the concentration should be measured for each batch. It must not exceed the maximum permitted level.

Every batch of vaccine should be tested for potency using a validated serological test that correlates with the protection obtained in the efficacy experiment, as described under Section C.2.c.ii. The potency test is not necessarily carried out in the target animal – mice or rabbits can be used. In these latter cases, correlation has to be shown with protective antibody levels in the target animal.

c) Requirements for authorisation ~~**Validation as a vaccine**~~

~~i) *Purity*~~

~~Both the master seed and the working seed must be pure cultures, free from bacterial, mycotic, mycoplasmal and viral contamination.~~

~~Identity of the bacterial species and the production of relevant antigens should be confirmed.~~

i) *Safety requirements*

Although inactivation of the bacterial cultures by a validated method is a standard procedure, both bacterial species produce dermonecrotic toxins; detoxification of these toxins should be confirmed when toxoids are used as vaccine components. Standard safety tests for inactivated vaccines should be carried out (see Chapter 1.1.8).

When an oil emulsion is used as the adjuvant, accidental injection of the operator can cause a severe local reaction. Medical attention should be sought immediately, treating the wound as a grease-gun injury.

ii) *Efficacy requirements*

Normally the vaccine is applied during the late stage of pregnancy, so that progeny will be protected by the uptake of colostral antibodies. The efficacy of a trial vaccine should be measured by vaccinating groups of pregnant sows. Their progeny should be challenged by virulent cultures of *B. bronchiseptica* and toxin-producing *P. multocida*. Significant protection should be obtained against the clinical signs of the progressive form of atrophic rhinitis, i.e. turbinate atrophy. The clinical signs induced in the controls and vaccinates may be compared according to the scoring system of Done (1976).

When the vaccine may be applied irrespective of the stage of pregnancy, duration of immunity should be at least 6 months, so that booster vaccinations twice a year should maintain effective antibody levels.

iii) *Stability*

Every batch of vaccine should be subjected to an accelerated shelf-life test, which has been correlated with real-time shelf-life testing.

3. Vaccines based on biotechnology

A number of vaccines incorporating enzymatically inert *P. multocida* toxin subunits or whole toxin rendered inactive through mutation have been described and evaluated under experimental conditions. The recombinant proteins are generally expressed in *Escherichia coli* and purified prior to use. At present, only a few vaccines that include recombinant toxin or toxin subunits are commercially available; they may or may not include a *P. multocida* bacterin. Evidence so far suggests such vaccines elicit levels of toxin-specific antibody equal to or greater than those obtained with vaccines containing native, chemically inactivated toxoid.

~~2. Method of manufacture~~

~~Both *B. bronchiseptica* and *P. multocida* cultures should be propagated in media that supports efficient growth and allows optimal expression of the antigens that are relevant for the induction of protective antibodies. *Bordetella bronchiseptica* should be confirmed to be a phase I culture and, for *P. multocida*, it should be confirmed that the culture contains sufficient levels of toxin.~~

~~*Bordetella bronchiseptica* cells, and either *P. multocida* cells and/or toxin, are inactivated, detoxified and formulated with an adjuvant. Commonly used adjuvants are aluminium salts or oil emulsions.~~

~~3. In-process control~~

~~During the manufacturing process, the following in-process controls are carried out.~~

~~a) Purity and identity of the seed cultures~~

~~Cultures are inoculated on blood agar plates and incubated. No nonspecific colonies should grow on these plates.~~

~~b) Purity and identity of the production cultures~~

~~Cultures are inoculated on blood agar plates and incubated. No nonspecific colonies should grow on these plates.~~

~~c) Inactivation of cultures before further processing~~

~~Cultures are inactivated with formaldehyde. Tests are performed to check the effectiveness of the inactivation process and to test for residual formaldehyde.~~

~~d) Quantification of antigens~~

~~This is carried out by performing a total cell count using a bacterial counting chamber for enumerating whole cells or an antigenic mass determination for defined antigens, e.g. *P. multocida* toxin, by quantitative enzyme immunoassay.~~

~~4. Batch control~~

~~a) Sterility~~

~~Every batch of vaccine should be tested for sterility according to standard methods (see Chapter 1.1.9 Tests for sterility and freedom from contamination of biological materials) described in the European Pharmacopoeia or the United States Code of Federal Regulations.~~

~~b) Safety~~

~~Every batch of vaccine should be tested for safety in the target animal, by giving a double dose by the recommended route of vaccination, and a second, single dose 2 weeks later. No abnormal local or systemic reactions should occur.~~

~~c) Potency~~

~~Every batch of vaccine should be tested for potency using a validated serological test that correlates with the protection obtained in the efficacy experiment, as described under Section C.1.c.iii. The potency test is not~~

~~necessarily carried out in the target animal – mice or rabbits can be used. In these latter cases, correlation has to be shown with protective antibody levels in the target animal.~~

~~**d) Duration of immunity**~~

~~Normally the vaccine is applied during the late stage of pregnancy, so that progeny will be protected by the uptake of colostral antibodies.~~

~~When the vaccine may be applied irrespective of the stage of pregnancy, duration of immunity should be at least 6 months, so that booster vaccinations twice a year should maintain effective antibody levels.~~

~~**e) Stability**~~

~~Every batch of vaccine should be subjected to an accelerated shelf-life test, which has been correlated with real time shelf life testing.~~

~~**f) Preservatives**~~

~~When a preservative is used, the concentration should be measured for each batch. It must not exceed the maximum permitted level.~~

~~**g) Precautions**~~

~~When an oil emulsion is used as the adjuvant, accidental injection of the operator can cause a severe local reaction. Medical attention should be sought immediately, treating the wound as a grease gun injury.~~

~~**5. Tests on the final product**~~

~~**a) Safety**~~

~~Every batch of vaccine should be tested for safety, as described in Section C.4.b.~~

~~**b) Potency**~~

~~Every batch of vaccine should be tested for potency, as described in Section C.4.c.~~

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