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

SECTION 1.1.

INTRODUCTORY CHAPTERS

CHAPTER 1.1.1.

COLLECTION, SUBMISSION AND ~~SHIPMENT~~ STORAGE OF DIAGNOSTIC SPECIMENS

INTRODUCTION

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

Specimens ~~for laboratory testing~~ must be collected using appropriate biosafety and ~~laboratory~~ containment measures in order to prevent contamination of the environment, animal handlers, and individuals doing the sampling as well as to prevent cross-contamination of the specimens themselves. Care should additionally be taken to avoid undue stress or injury to the animal and physical danger to those handling the animal. Biological materials should be packaged to rigorously control for leakage, and then labelled with strict adherence to the applicable regulations guiding their transport as outlined in Chapter 1.1.2.

A. COLLECTION OF SAMPLES

1. General considerations

Careful consideration must be given to the collection, containment, and ~~transport~~ storage of the specimens, including biosafety measures that must be in place to prevent contamination of the environment or exposure of other animals and humans to potentially infectious materials (see Chapter 1.1.3 *Standard for Managing Biorisk in Veterinary Laboratories*). For information on transport of specimens see Chapter 1.1.2 *Transport of Specimens of Animal Origin*.

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

The purposes of testing will be aligned with the purposes for which tests are validated, as listed in Chapter 1.1.5 *Principles and methods of validation of diagnostic assays for infectious diseases*, namely:

i) Demonstration of freedom from infection in a defined population.

ii) Certification of freedom from infection or presence of the agent in individual animals or their products for trade/movement purposes.

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

iv) Confirmatory diagnosis of suspect or clinical cases.

v) Estimation of prevalence of infection or exposure to facilitate risk analysis.

vi) Determination of immune status of individual animals or populations (post-vaccination).

Epidemiologically appropriate sampling plans should be developed prior to collection of specimens, as described in Section B and Appendix 1.1.1.1. These will specify the number of animals or other sampling units to be sampled.

Specimens must be collected according to a sound knowledge of the epidemiology and pathogenesis of the disease under investigation, or the disease syndrome to be diagnosed. This will lead to the sampling of tissues or fluids most likely to contain the infectious agent or evidence of the infection. Considerations will include the tissue predilection(s) or target organ, the duration and site of infection in each tissue type and the duration and route of shedding, or the time frame in which evidence of past infection, such as an antibody response, can be detected reliably by the tests to be deployed. These considerations will also indicate the method(s) of collection to be used. In many herd or flock-based disease investigations it is beneficial to collect specimens from a healthy cohort for comparative epidemiological or baseline testing (e.g. case-control and cohort approaches for diagnostic testing) and for validation purposes.

Where chemical euthanasia or anaesthesia is required for animal restraint, the impact of the chemical on the test result (e.g. toxicology testing) must be considered. Some laboratory tests are not compatible with specific blood anticoagulants and tissue preservatives, such as heparin, formalin, dry ice (exposure of the test sample to elevated levels of CO_2), or even freezing. While it is critical to collect specimens as aseptically as possible, equal care must be taken to avoid contamination with detergents and antiseptic treatments used to clean the collection site on the animal, as these agents may interfere with the laboratory test procedures. Procedures requiring tissue culture of pathogens, as well as many molecular-based tests, can be negatively affected by chemicals or detergents commonly used in the manufacture or preparation of collection tools (e.g. chemicals used in manufacture of some types of swabs and detergents used in cleaning glassware).

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

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

Where investigating diseases of unknown cause multiple different ~~tissues~~ specimens that represent the different stages of the disease progression in an animal or the population of animals (e.g. the pre-clinical, early clinical, active clinical, chronically affected and convalescent phases) should be collected. Epidemiological considerations for sampling are particularly critical for diagnosing population-related diseases, as would occur with beehives, flocks, and herds. Epidemiological principles used for sampling are further introduced in Section B of this chapter.

Specimens can be collected ante-mortem or post-mortem. Specific considerations regarding different specimen types are as outlined below.

2. Blood

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

the collected blood be thoroughly mixed with the chosen anticoagulant during or immediately following its sampling from the animal.

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

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

3. Faeces

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

4. Epithelium

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

5. Ocular sampling

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

6. Sampling the reproductive tract

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

7. Nasal discharge, saliva, and vesicular fluids

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

8. Milk

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

9. Tissues collected at necropsy

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

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

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

For fresh specimens particular attention must be paid to their handling and storage to ~~keep~~ avoid autolysis and overgrowth by bacterial and fungal contaminants ~~to a minimum~~. Ideally, freshly collected specimens are ~~collected into containers immediately stored in a temperature controlled storage system and~~ kept at a constant cool temperature ~~during transport to the laboratory and~~ from collection until processing for testing. Where such a cold chain cannot be provided fresh specimens for some test procedures can be collected into fluids such as ethylene glycol that inhibit the growth of secondary organisms. Where such strategies are compatible with the subsequent test methods the option is mentioned in the relevant chapters of the *Terrestrial Manual* for each disease.

Preservation of post mortem specimens is most frequently achieved by collection into formalin solution. Where such chemical fixative is supplied to pathology staff by laboratories or competent authorities they must ensure

adequate training in health and safety aspects of the use of such chemicals and training in compliance with regulations relating to the transport of such chemicals.

9. Environment and feed

Environmental sampling may be of litter, bedding, water from troughs and drinkers, or feed which has been exposed to urine, faeces, and/or saliva of affected animals, or swabbed surfaces of facilities, ventilation ducts, drains or feed containers. If specialised equipment is available circulating air may be sampled.

10. Honey bees

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

B. EPIDEMIOLOGICAL APPROACHES TO SAMPLING

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

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

To detect evidence of infection in line with the other five purposes of testing as listed in Section A above the sampling should be done within the context of a surveillance programme. The criteria for the design and implementation of effective surveillance are described in Chapter 1.4 *Animal health surveillance* of the *Terrestrial Animal Health Code* (*Terrestrial Code*). Identification of animals for sampling in surveillance programmes may be targeted (risk-based) or random. Risk-based sampling based on epidemiological knowledge of the infection under study or on epidemiological observations of the population under study is intended to result in the most likely detection of infected individuals.

Inferences on the status of a population such as estimation of prevalence, immune status or disease freedom should be based on random sampling. Random sampling ensures that the animals sampled are representative of the population, within the practical constraints imposed by different environments and production systems. Additionally, random sampling allows extrapolation of the findings of the study to the overall population (with an appropriate confidence interval). The epidemiological principles for sample size estimation are addressed in Appendix 1.1.1.1.

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

Sampling and laboratory testing may also be used in support of epidemiologically based diagnostics and studies such as case control, structured longitudinal, and cohort studies (Fosgate & Cohen, 2008; Mann, 2003; Pfeiffer, 2010). The number and selection of the animals to be sampled and the nature of the specimens will be part of the study design.

C. INFORMATION TO BE SENT WITH SPECIMENS

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

Information regarding the location and contact information of the submitter and the premises sampled, the case information and associated epidemiological information, as detailed below, should always accompany the specimens to the laboratory. Such documentation should be placed in a plastic envelope on the outside of the shipping container so as to be available for reference during transport and should also be duplicated inside the shipping container between the secondary and the outer packaging (see also Chapter 1.1.2 *Transport of specimens of animal origin*). It would be advisable to contact the receiving laboratory to obtain an appropriate submission form and other relevant shipping and handling information.

Necessary information includes:

1. Location and contact information

i) Name and address of owner/occupier of the animal owner and/or the sampled premises and the geolocation (latitude and longitude, if available) where disease occurred, with appropriate contact information (telephone and fax numbers, e-mail address).

ii) Name, postal and e-mail address, telephone and fax numbers of the sender.

2. Case information

i) Disease agents suspected and tests requested.

ii) Species, breed, sex, age and identity of the animals sampled, and trackability number when available.

iii) Date samples were collected and submitted.

iv) List and type of samples submitted with transport media used.

v) Case history:

a) The clinical signs and their duration including the temperature of sick animals, condition of mouth, eyes and feet, and milk or egg production data as relevant.

b) A list and description of the animals examined and the findings of the ante- and post-mortem examinations.

c) The length of time sick animals have been on the premise; if they are recent arrivals, from where did they originate.

d) The date of the first cases and of subsequent cases or losses, with, for tracking, any appropriate previous submission reference numbers.

3. Epidemiological information

i) A description of the spread of infection in the herd or flock.

ii) The number of animals on the premise by species, the number of animals dead, the number showing clinical signs, and their age, sex and breed.

iii) The type and standard of husbandry, including biosecurity measures and other relevant factors potentially associated with the occurrence of cases.

iv) History of foreign travel by owner or of introduction of animals from other countries or regions.

v) Any medication given to the animals, and when given.

vi) Vaccination history describing the type of vaccines used and dates of application.

vii) Other observations about the disease, husbandry practices and other disease conditions present.

D. RECEIPT, STORAGE AND ARCHIVES OF LABORATORY SUBMISSIONS

1. Reception of samples

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

A risk assessment (RA) as outlined in chapter 1.1.3 should be performed before systems for handling biological agents and toxins are established in order to define the appropriate biosafety and laboratory biosecurity measures. The RA should lead to the development of stated policy and procedures for the operation of the whole process of receiving submissions to the laboratory.

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

a) Specimen reception area

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

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

b) Submission unpacking, registration and preparation for further processing

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

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

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

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

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

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

c) Emergencies

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

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

2. Storage and archives

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

Materials routinely needed as reference standards and for assay validation are described in Chapter 1.1.4 *Quality management in veterinary testing laboratories*. The materials maintained in laboratory archives should be representative of the agents handled and the types of samples used in the different testing methods performed, which would variously include fresh and fixed tissues and fluids, paraffin-embedded tissues, and stabilised or otherwise preserved cultures. The World Federation for Culture Collections (WFCC: *www.wfcc.info/collections*) is a useful source of information and reference documentation for developing, maintaining, and sharing culture collections, and has published a comprehensive guide for establishing and operating microbial culture collections (WFCC, 2010). It is part of the remit of OIE Reference Laboratories to supply reference materials.

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

a) Stabilisation and storage

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

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

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

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

b) Documentation and Inventory

Agents and tissues maintained in an archive must be correctly identified and sufficient supporting data that characterises the sample or agent must be recorded. For reference materials, further documentation that

authenticates the agent or tissue is required. The unique identity of the tissue, fluid, or agent and the storage location are best maintained in an electronic or paper inventory record which also documents the date the material was obtained, date and method of preservation, volume of material stored, source of the material including associated species, geographical location, and the clinical history of the donor animal and the disease situation of the flock or herd. Additional information is extremely useful and generally includes the original method of isolation/recovery, characterisation (e.g. available data on biochemical properties, antibody or antigen titre, and genetic sequence) as well as additional history on handling of the material (e.g. number of passages for infectious agents and cell lines, dates the archived material was frozen-thawed or rehydrated and the dates it was transferred to different storage conditions or containers). Inventories are most often organised by assigning a unique identifying number or alphanumeric code to each sample (sample container) that is cross-referenced to a database or inventory log. Inventory records can be manual data logs, computerised spreadsheets, or specialised computer programs. However the records are managed, they must be kept current and the information entered must be traceable to its source. The identification of the individual making an entry or modification to the sample inventory should be recorded.

c) Biosafety and laboratory biosecurity

As a first step in establishing an archive, a laboratory biorisk assessment addressing biosafety and laboratory biosecurity issues, including any control or mitigation measures to be implemented, must be completed. As further defined in chapter 1.1.3, an appropriate risk assessment for archived samples should address the technical competency needed of staff handling the tissues, fluids, and agents, with particular emphasis on those materials that are potentially infectious or toxic to workers, animals, and the environment in and around the laboratory. The laboratory should consider all biorisk management measures needed to protect the integrity of the sample, as well as the health of the workers and environment, from the time the original sample is received through the long-term storage and ultimate use or destruction of the material(s).

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

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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

APPENDIX 1.1.1.1.

EPIDEMIOLOGICAL APPROACHES FOR SAMPLING: SAMPLE SIZE CALCULATIONS

The type and number of samples needed depends on the desired purpose. Sample size calculation for each of the main purposes of testing, where random sampling has been used, may be approached as follows:

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

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

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

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

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

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

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

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

In the case of testing individual animals, it is generally expected that all animals will be tested. The critical question in this case is related to the negative predictive value (NPV) of the test and the probability of having at

least one false negative individual in a group. The negative predictive value of a test is defined as the probability that an animal is not infected given that it tested negative. The NPV is a function of the diagnostic sensitivity and specificity of the test(s) used and the prevalence of the infection in the population where the animals come from. In general, the probability of having at least one false negative in a group is calculated as:

The negative predictive value is calculated as follows:

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

Where:

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

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

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

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

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

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

5. Estimate prevalence of infection

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

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

Where
n: is the required simple size
Z: is the value of the Z distribution for the desired confidence level (usually 95%)
p: is the expected prevalence in the population

q: is 1-p
L: is the level of precision (or acceptable error)

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

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

Therefore, 289 animals would have to be randomly sampled.

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

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

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

a) Estimating the proportion of immune animals in a group

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

b) Estimating immunity at a defined threshold

- Expected prevalence (of immune animals) 80%, i.e. 400 out of 500 animals

- Confidence level 95%
- Perfect diagnostic sensitivity

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

On-line resources

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

Free Calc - http://www.ausvet.com.au/content.php?page=software#freecalc

Win Episcope: http://www.clive.ed.ac.uk/cliveCatalogueItem.asp?id=B6BC9009-C10F-4393-A22D-48F436516AC4

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* *

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CHAPTER 1.1.2.

TRANSPORT OF SPECIMENS OF ANIMAL ORIGIN

INTRODUCTION

The transport of infectious substances is covered by international regulations that are updated on a regular basis and are widely accessible via the internet, or through commercial and regulatory transportation affiliates. The World Health Organization (WHO) guidance document on "Transport of Infectious substances" summarising the different transport regulations is regularly updated. This chapter is based on international regulations and adapted accordingly, to best cover the transport requirements for veterinarians transporting samples from the field to laboratories, as well for transportation between veterinary laboratories within a country. Practical explanation on how to transport biological substances according to the specific dangerous goods transport regulations will be explained in this chapter.

This chapter will focus on the transport of specimens that are non-hazardous for humans or animals or where there is a minimal likelihood that pathogens are present (exempt specimens). It will briefly touch on infectious substances, including diagnostic materials and is based on international transport regulations. Specific examples for veterinary laboratories are provided.

The international regulations for the transport of infectious substances by any mode of transport are based upon the Recommendations made by the Committee of Experts on the Transport of Dangerous Goods (UNCETDG), a committee of the United Nations Economic and Social Council. The Recommendations are presented in the form of Model Regulations covering rail, road, sea and post.

A. BASIC PRINCIPLES

In the interest of veterinary public health, animal specimens must be transported safely, timely, efficiently and legally from the place where they are collected to the place where they are analysed. The collection of specimens from animals in the field is covered in Chapter 1.1.1 *Collection, submission and storage of diagnostic specimens*. All specimens should be packaged and transported in accordance with local, national and international regulations. ~~regardless of the distance, in such a way as to~~ The procedures should minimise the risk of exposure for those engaged in transportation and should protect the environment and susceptible animal populations from potential exposures. Additionally inefficient packaging that allows for damage or leakage will likely delay the delivery of the shipment to the laboratory, delaying or preventing critical laboratory analyses from being performed. Specimens should always be packaged and transported to protect the integrity of the specimens, as well as to avoid cross-contaminating other specimens. Minimal requirements for the transport of specimens follow the principle of triple packaging, consisting of three layers as described below:

- *Primary inner receptacle:* A primary watertight, leak-proof or stiff-proof receptacle containing the specimen. The receptacle is packaged with enough absorbent material (e.g. cellulose wadding, paper towels, house hold paper, cotton balls) between the primary and the secondary container to absorb all fluid in case of breakage. Even though the regulations do not prohibit glass, primary receptacles should preferably not be breakable. In addition, they should not contain any sharps (e.g. vacutainer with needle), particularly when using soft secondary and outer containers.

- *Secondary packaging:* A second durable, watertight, leak-proof packaging to enclose and protect the primary receptacle(s) (e.g. sealed plastic bag, plastic container, screw-cap can). Several cushioned primary receptacles may be placed in one secondary packaging, but sufficient additional absorbent material shall be used to absorb all fluid in case of breakage.

- *Outer packaging:* Secondary packaging is placed in outer shipping packaging (e.g. sturdy cardboard box, rigid cooler) with suitable cushioning material. Outer packaging protects the contents from outside influences, such as physical damage, while in transit.

Biological materials should be prepared for shipment by personnel that are trained and competent in packaging procedures and also knowledgeable of the shipping requirements and regulations. Whenever possible, specimens should be directly transported to the laboratory to ensure a rapid and reliable system using individuals that are trained and competent in the shipping and transportation process. The laboratory receiving the specimens must be informed in advance of the time and mode of the arrival of the specimens in order to be prepared to receive the specimen.

B. DEFINITIONS OF SPECIMENS TO BE TRANSPORTED

Definitions are based on the United Nations Model Regulations and are italicised.

1. Infectious substances

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

a) Category A

An infectious substance which is transported in a form that, when exposure to it occurs, is capable of causing permanent disability, life-threatening or fatal disease in otherwise healthy humans or animals. Indicative examples of substances that meet these criteria are given in the table A.

Note: Some organisms are considered Category A only when in culture form. New or emerging pathogens that do not appear on the list but meet the criteria, must also be transported as Category A.

b) Category B

An infectious substance which does not meet the criteria for inclusion in Category A.

Most laboratory submissions (apart from exempt specimens – see below) fall into this category. The official nomenclature for shipping is “Biological Substance, Category B” (formerly “Diagnostic Specimens”)

2. Cultures

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

3. Patient specimens

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

4. Biological products

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

5. Genetically modified micro-organisms (GMMOs) and organisms (GMOs)

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

6. Medical or clinical wastes

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

~~7. CITES~~

~~Convention for the International Trade in Endangered Species~~

8. Responsibilities

The efficient transport and transfer of substances requires co-ordination between the sender, the carrier and the receiver to ensure that the material is transported safely and arrives on time and in good condition.

It is the responsibility of the ~~shipper~~ sender to ensure the correct classification, packaging, labelling and documentation of all substances destined for transport.

a) The sender (shipper, consignor)

i) Makes advance arrangements with the receiver including investigating the need for import/export permits;

ii) Makes advance arrangements with the carrier to ensure:

a) that the shipment will be accepted for appropriate transport;

b) that the shipment (direct transport if possible) is undertaken by the most direct routing;

iii) Prepares necessary documentation, including permits, dispatch and shipping documents if necessary;

iv) Notifies the receiver of transportation arrangements once these have been made, well in advance of the expected arrival time.

b) The carrier

i) Provides advice to the sender regarding the necessary shipping documents and instructions for their completion;

ii) Provides advice to the sender about correct packaging;

iii) Assists the sender in arranging the most direct routing and then confirms the routing and provides, if possible, ways to track the parcel;

iv) Maintains and archives the documentation for shipment and transport.

c) The receiver (consignee)

i) Obtains the necessary authorisation(s) from national authorities for the importation of the material;

ii) Provides the sender with the required import permit(s), letter(s) of authorisation, or other document(s) required by the national authorities;

iii) Arranges for the most timely and efficient collection on arrival;

iv) Should acknowledge receipt to the sender.

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

9. Exemptions

Judgment by trained and competent laboratory professionals is required to determine if samples to be shipped are qualified as hazardous to humans or to animals or are exempt for shipping purposes. That judgment should be based on the known medical history of the animal(s), signs and individual circumstances of the specimen source, and endemic local disease conditions.

Specimens that do not contain infectious substances are not subject to dangerous goods regulations.

Substances containing micro-organisms that are non-pathogenic to humans or animals are not subject to dangerous goods regulations, unless they meet the criteria for inclusion in another class.

Substances in a form in which any pathogens present have been neutralised or inactivated such that they no longer pose a health risk are not subject to dangerous goods regulations, unless they meet the criteria for inclusion in another class.

Environmental specimens (*including food and water specimens*) *that are not considered to pose a significant risk of infection are not subject to dangerous goods regulations, unless they meet the criteria for inclusion in another class.*

Dried blood spots, collected by applying a drop of blood onto absorbent material, or faecal occult blood screening tests are not subject to dangerous goods regulations.

Human or animal specimens for which there is minimal likelihood that pathogens are present are not subject to dangerous goods regulation if the specimen is carried in a packaging which will prevent any leakage (*three layer principle, see Section A. Basic principles*) *and which is marked with the words "Exempt human specimens" or "Exempt animal specimens", as appropriate.*

Examples of specimens in the veterinary field *which may be transported as exempt* include specimens from surveillance studies, export controls of healthy animals (e.g. certification of freedom from classical swine fever) or determination of immune status of individual animals or populations (post-vaccination).

Samples containing DNA, RNA or plasmids (except prions) are not covered by the dangerous goods transport regulation. If these specimens are shipped in liquid form it is recommended to use the packaging system described below. If they are shipped on filter paper it can be shipped as regular mail. There may be specific regulations in place in some countries for the shipment, export or import of nucleic acids.

If it is likely that pathogens present in the specimens can cause harm to the human or animal population if exposed, then they must be assigned either to category A or B.

a) Packaging for exemptions

Specimens should always be packaged and transported to protect the integrity of the specimens. The basic principle of the three-layer system may apply for these specimens as well.

The three-layer system consists of the following elements:

i) *a leak-proof primary receptacle*(*s*) (*avoid glass containers*);

ii) *a leak-proof secondary packaging* (*e.g. plastic container or tight plastic bag*); *and*

iii) *an outer packaging of adequate strength for its capacity, mass and intended use, and with at least one surface having minimum dimensions of 10 cm × 10 cm;* (*e.g. plastic envelope or box, cardboard box, plasticised paper envelope*). *Paper envelopes for letters are not considered as suitable outer packaging*.

For liquids, absorbent material in sufficient quantity to absorb the entire contents should be placed between the primary receptacle(*s*) *and the secondary packaging so that, during transport, any release or leak of a liquid substance will not reach the outer packaging and will not compromise the integrity of the cushioning material.* Different types of absorbent material can be used (e.g. paper towels, household paper, toilet paper or any other suitable material).

b) Documentation and marking

The submission form, with information and case history, sent with the specimens should be placed in a plastic bag between the secondary and the outer packaging and on the outside of the consignment. Further guidance on information to be sent with the specimens can be found in chapter 1.1.1.

Individual specimens should be clearly identified using appropriate methods. Markings should withstand the condition of use, i.e. being wet or frozen. Attached plastic labels will fall off if stored at –70°C or use of wrong pencils may rub off containers.

The outer package of samples from *animal specimens for which there is minimal likelihood that pathogens are present* should be marked with the words *"Exempt animal specimen".*

NOTE: *For air transport, packagings for specimens exempted under this paragraph shall meet the packaging and marking described above.*

C. TRANSPORT OF INFECTIOUS SUBSTANCES, CATEGORY A (UN 2814 OR UN 2900)

Due to the nature of category A specimens which contain highly hazardous micro-organisms, more stringent packaging and transport regulations apply. Category A substances, although not necessarily a human pathogen, may have a high economic or trade impact on specific countries should there be release to the environment. Therefore other infectious substances may be added to this list by individual countries (e.g. cultures of Newcastle disease virus where the virus is exotic to the country or region). Furthermore, for those micro-organisms listed as category A infectious substances (cultures only), patient specimens of these pathogens do not require category A transport practices. For these specimens Category B transport practices should be applied.

Due to the highly hazardous nature of the Category A samples the packaging must meet special requirements. The principle of three layers also applies here, and the transport containers and outer packaging must meet the criteria defined in the relevant regulations, which can be found in Section J (e.g. the packaging must be UN certified and must have passed specific tests). The packages are marked to provide information about the contents of the package, the nature of the hazard and the packaging standards applied (e.g. "*INFECTIOUS SUBSTANCE, AFFECTING HUMANS*; UN 2814" or "*INFECTIOUS SUBSTANCE, AFFECTING ANIMALS ONLY*; UN 2900").

Furthermore, personnel packing, shipping or transporting Category A specimens are required to be specially trained and certified according to international regulations. This approval typically involves attendance at approved courses and passing of examinations.

Table A: *Examples of infectious substances included in Category A (indicative list)*

Indicative examples of infectious substances included in Category A in any form unless otherwise indicated	
UN number and proper shipping name	**Micro-organism**
UN 2814 Infectious substance, affecting humans	*Bacillus anthracis* (cultures only)
	Brucella abortus (cultures only)
	Brucella melitensis (cultures only)
	Brucella suis (cultures only)
	Burkholderia mallei – Pseudomonas mallei – glanders (cultures only)
	Burkholderia pseudomallei – Pseudomonas pseudomallei (cultures only)
	Chlamydia psittaci – avian strains (cultures only)
	Clostridium botulinum (cultures only)
	Coccidioides immitis (cultures only)
	Coxiella burnetii (cultures only)
	Crimean-Congo haemorrhagic fever virus
	Dengue virus (cultures only)
	Eastern equine encephalomyelitis virus (cultures only)
	Escherichia coli, verotoxigenic (cultures only)[1]
	Ebola virus

Indicative examples of infectious substances included in Category A in any form unless otherwise indicated	
UN number and proper	**Micro-organism**

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

shipping name	
UN 2814 Infectious substance, affecting humans	Flexal virus
	Francisella tularensis (cultures only)
	Guanarito virus
	Hantaan virus
	Hantaviruses causing haemorrhagic fever with renal syndrome
	Hendra virus
	Hepatitis B virus (cultures only)
	Herpes B virus (cultures only)
	Human immunodeficiency virus (cultures only)
	Highly pathogenic avian influenza virus (cultures only)
	Japanese Encephalitis virus (cultures only)
	Junin virus
	Kyasanur Forest disease virus
	Lassa virus
	Machupo virus
	Marburg virus
	Monkeypox virus
	Mycobacterium tuberculosis (cultures only)[1]
	Nipah virus
	Omsk haemorrhagic fever virus
	Poliovirus (cultures only)
	Rabies virus (cultures only)
	Rickettsia prowazekii (cultures only)
	Rickettsia rickettsii (cultures only)
	Rift Valley fever virus (cultures only)
	Russian spring-summer encephalitis virus (cultures only)
	Sabia virus
	Shigella dysenteriae type 1 (cultures only)[2]
	Tick-borne encephalitis virus (cultures only)
	Variola virus
	Venezuelan equine encephalitis virus (cultures only)
	West Nile virus (cultures only)
	Yellow fever virus (cultures only)
	Yersinia pestis (cultures only)

200

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

201

Indicative examples of infectious substances included in Category A in any form unless otherwise indicated	
UN number and proper shipping name	**Micro-organism**
UN 2900 Infectious substance, affecting animals only	African swine fever virus (cultures only)
	Avian paramyxovirus Type 1 – Velogenic Newcastle disease virus (cultures only)
	Classical swine fever virus (cultures only)
	Foot and mouth disease virus (cultures only)
	Lumpy skin disease virus (cultures only)
	Mycoplasma mycoides – contagious bovine pleuropneumonia (cultures only)
	Peste des petits ruminants virus (cultures only)
	Rinderpest virus (cultures only)
	Sheep-pox virus (cultures only)
	Goatpox virus (cultures only)
	Swine vesicular disease virus (cultures only)
	Vesicular stomatitis virus (cultures only)

202 ## D. TRANSPORT OF BIOLOGICAL SUBSTANCES, CATEGORY B (UN3373)

203 Samples containing micro-organisms which do not cause life-threatening disease to humans or animals can be
204 assigned to Category B and are assigned the identification number UN3373.

205 Some examples for Category B shipments are given below:

206 Typically a specimen with a high likelihood to contain pathogenic organisms shipped for disease diagnosis (e.g.
207 confirmatory diagnosis of suspect or clinical cases, specimens for differential diagnosis, blood samples for
208 classical swine fever or sheep pox diagnostics or throat samples from chickens for avian influenza) can be
209 assigned to Category B. Although specimens can be shipped as Category B, pure cultures of the biological agent,
210 such as classical swine fever or sheep pox (see list of micro-organisms of Category A) must follow the the
211 requirements of Category A (UN 2900, infectious substances affecting animals only) due to the infectious nature
212 of the specific organism.

213 Alternately, shipments of cultures of less pathogenic agents, e.g. bovine virus diarrhoea (BVD), *Salmonella*
214 *enteritidis, Salmonella typhimurium* or *Listeria monocytogenes* can be assigned to Category B.

215 The following description of the packaging and labelling are a summary of the requirements for surface transport.
216 For international shipment and air transport additional requirements do apply. The exact details can be found in
217 packaging instruction P650 (see Section J).

218 ### 1. Packaging

219 The triple packaging system continues to apply, including for local surface transport (description: see Section A.
220 *Basic principles*).

221 The packaging shall be of good quality, strong enough to withstand the shocks and loadings normally
222 encountered during carriage, including trans-shipment between vehicles or containers, as well as any removal
223 from an overpack (several packages combined into a single shipment). The smallest overall external dimension
224 shall be 10 × 10 cm.

225 For surface transport either the secondary packaging or the outer packaging must be rigid.

226 ~~*For air transport only:*~~

i) For air transport, the primary receptacle or secondary packaging must be capable of withstanding, without leakage, an internal pressure of 95 kPa in the range of –40°C to 55°C;

ii) for liquids: no primary receptacle shall exceed 1 litre and the outer packaging must not contain more than 4 litres;

iii) for solids: the outer packaging must not contain more than 4 kg. This restriction doesn't apply for body parts, organs and whole bodies. The three layer principle has to be adopted accordingly using appropriate packaging systems.

iv) the entire package should be able to withstand being dropped from a distance of 1.2 metres (4 feet) without damage to or leakage from the content.

2. Marking

The package must display the proper labelling to guarantee safe delivery in time at the correct destination.

Label is as follows:

i) Packages should be clearly labelled with the delivery address and sender's details with emergency contact details including named persons with telephone numbers for both the ~~shipper~~ sender and the recipient.

II) Mark with the proper shipping name in letters at least 6 mm high: BIOLOGICAL SUBSTANCE, CATEGORY B (Figure 1)

iii) In addition to the shipping name the marking shown below (UN3373 diamond) is used for shipments of Category B infectious substances. If a biohazard label is not present on the primary or secondary packaging, it must be present on the outer packaging.

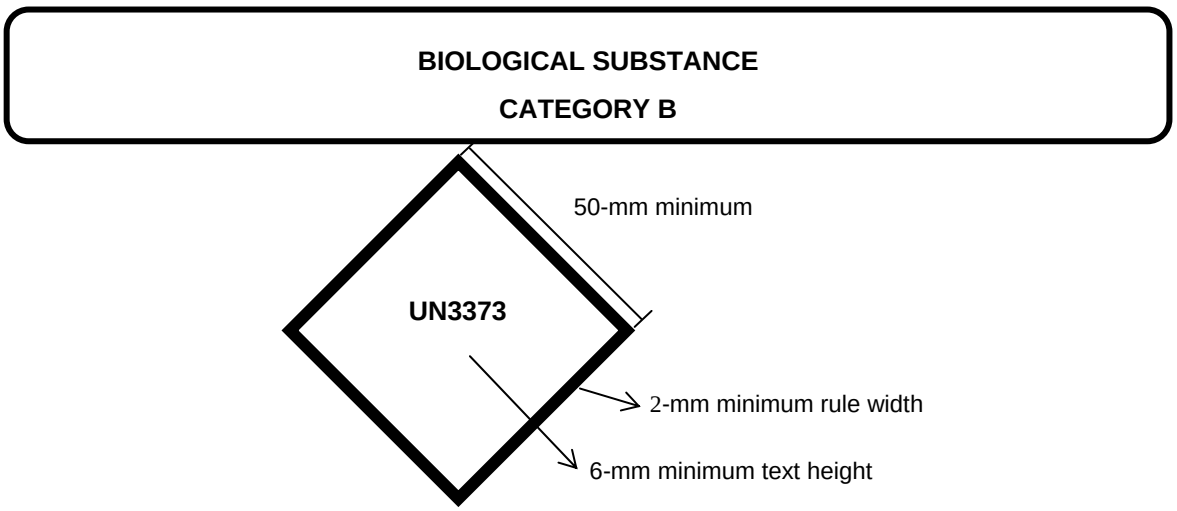

Figure 1: *UN3373 mark for the transport of Category B substances*

3. Documentation

There is no special documentation required for the shipment of Category B specimens for surface transport.

The information and case history sent with the specimens should be placed in a plastic bag between the secondary and the outer packaging and on the outside of the consignment. If a sample is shipped for diagnostic purposes further guidance on information to be sent with can be found in chapter 1.1.1.

For air transport only:

The documentation consists of the airway bill **for air transport** (form provided and filled out by ~~shipper~~ sender or carrier), showing "UN 3373", the text "BIOLOGICAL SUBSTANCE, CATEGORY B" and the number of packages in the "Nature and Quantity of Goods" box, and / or equivalent documents for the transport by road.

E. OVERPACKS

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

F. REFRIGERANTS

Refrigerants may be used to stabilise specimens during transport.

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

Dry ice must not be placed inside the primary or secondary receptacle because of the risk of explosions. A specially designed insulated packaging may be used to contain dry ice, typically a styropor or waxed-treated cardboard box to prevent leakage and maintain temperature. The packaging must permit the release of carbon dioxide gas if dry ice is used and the package (the outer packaging or the overpack) shall be marked "Carbon dioxide, solid" or "Dry ice" (see Section J). Dry ice is a dangerous good.

When using dry ice as refrigerant, the shipper must ensure that the class 9 safety label is shown on the upper half of each package with the number UN 1845. According to applicable transport regulations, only certified shippers are allowed to ship dry ice!

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

If liquid nitrogen is used as a refrigerant, additional requirements have to be followed according to the relevant regulations.

G. TRAINING

All personnel involved in the packaging, labelling and shipping of specimens should be appropriately trained and competent in packaging procedures and also knowledgeable of the shipping requirements and regulations.

H. EMERGENCY RESPONSES

Procedures for incidents such as spills or any other realistic and foreseeable emergencies should be part of the biorisk management system in order to response adequately to emergencies (see chapter 1.1.3).

I. SPECIAL CONSIDERATION FOR CITES

CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) is an international agreement between governments with the aim to ensure that international trade in specimens of wild animals and plants does not threaten their survival.

Some specimens to be transported from one country to another may be derived from species covered by CITES. Depending on the classification of the species, a CITES export permit or both export and import permits may be required and the appropriate documents have to be obtained. There is some variation of the requirements from one country to another and it is always necessary to check on the national laws that may be stricter.

Further information on CITES: http://www.cites.org/eng/disc/what.php

J. ~~SUMMARY OF~~ ADDITIONAL INFORMATION ON THE WORLD HEALTH ORGANIZATION, UNITED NATIONS AND OTHER INTERNATIONAL AND NATIONAL TRANSPORT GUIDANCE ~~ON TRANSPORT OF~~ FOR INFECTIOUS SUBSTANCES

Further reading and information

WHO Guidance on regulations for the "Transport of Infectious substances" 2011–2012, covering transport regulations on national and international and air transport by different means:

http://www.who.int/ihr/publications/who_hse_ihr_20100801/en/index.html

Swiss Expert Committee on Biosafety: "Transport, import and export of substances consisting of or containing pathogenic or genetically modified (micro)organisms"; practical explanation on how to transport biological substances according to the specific dangerous goods transport regulations

http://www.efbs.admin.ch/en/transport/index.html

Additional information on the United Nations System for the Transport of Dangerous Goods

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

http://www.unece.org/trans/danger/danger.htm

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

http://www.unece.org/trans/danger/publi/unrec/rev17/17files_e.html

The site below provides the full text of the European Agreement concerning the International Carriage of Dangerous Goods by Road (ADR) of 2009, and the amendments to ADR 2011, which entered into force on 1 January 2011~~ will enter into force on 1 January 2009~~, which can be downloaded in PDF format. Readers wishing to study the text relating to the transport of infectious substances should download Part 2 (2.2.62), Part 4 (search P620, P650) and Part 5:

http://www.unece.org/trans/danger/publi/adr/adr2011/11contentse.html

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

Air	ICAO: http://www.icao.int/Pages/default.aspx
Rail	RID: http://www.otif.org/. RID is primarily for the countries of Europe, North Africa and the Middle East. There are a number of countries (mainly Eastern Europe and Asia that apply RID through the Organization for Cooperation of Railways (OSJD); details of RID membership can be found at http://www.otif.org/en/about-otif/addresses-and-useful-links/member-states.html
Road	ADR: http://www.unece.org/trans/danger/publi/adr/country-info_e.htm (lists competent authorities)
Sea	IMO: http://www.imo.org
Post	UPU: http://www.upu.int/

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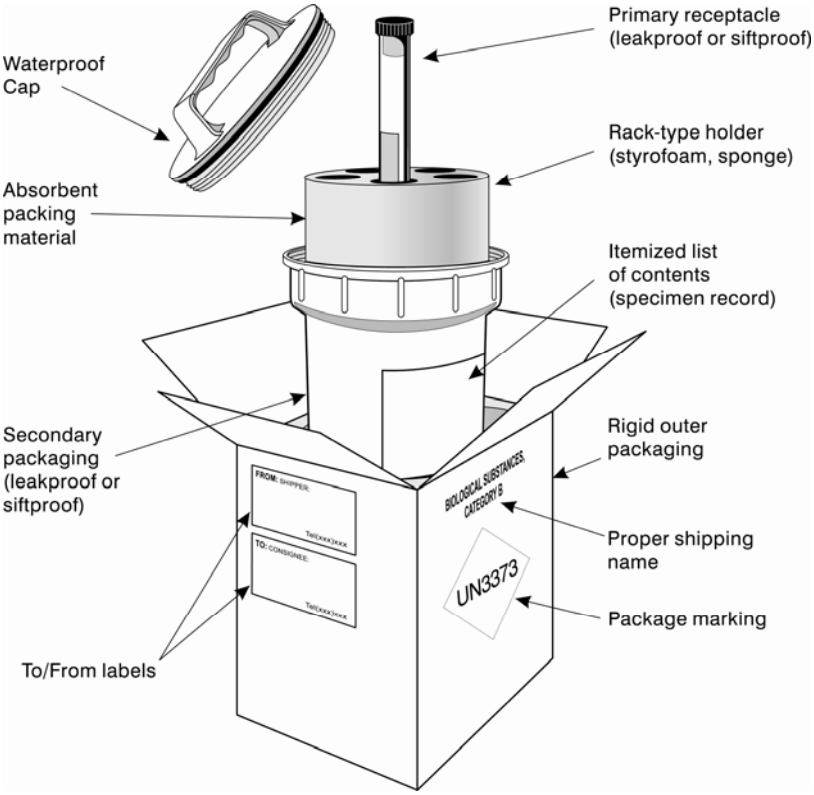
* *

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

APPENDIX 1.1.2.1.

EXAMPLE OF THE TRIPLE PACKAGING SYSTEM FOR THE PACKING AND LABELLING OF CATEGORY B

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



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

342 APPENDIX 1.1.2.2.

343 DECISION TREE FOR DECIDING ON THE 344 TRANSPORT REQUIREMENTS OF A SAMPLE

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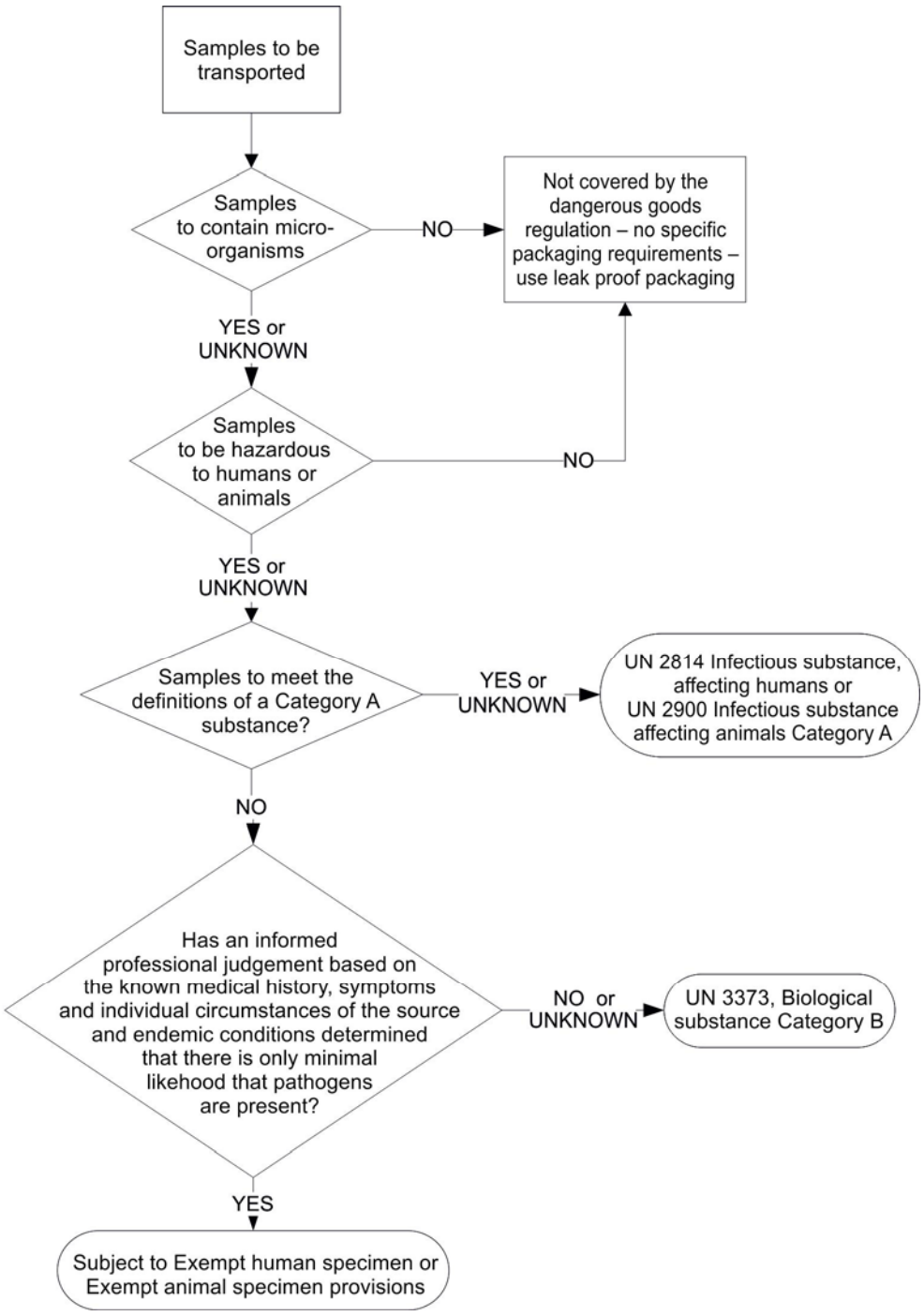

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CHAPTER 1.1.5.

PRINCIPLES AND METHODS OF VALIDATION OF DIAGNOSTIC ASSAYS FOR INFECTIOUS DISEASES

INTRODUCTION

Validation is a process that determines the fitness of an assay[1]*, which has been properly developed, optimised and standardised, for an intended purpose. All diagnostic assays (laboratory and field assays) ~~should be validated~~ for the species in which they will be used. 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)"*[2]*. To maintain a validated assay status, however, it is necessary to carefully monitor the assay's ~~daily~~ performance under conditions of routine use, often by tracking the behaviour of ~~internal~~ assay 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(s). Thus, a validated assay is continuously assessed to assure it maintains its fitness for purpose through both the assessment of results of the assay ~~internal~~ controls included with each run and through on-going assessment ~~of the assay~~ during routine use in the targeted population.*

~~*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, contributing to eradication of 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 more 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 required validation criteria (definition of intended purpose[s], optimisation, and standardisation) that must be assessed in order to achieve a validated assay, comprise steps in the assay development process. Accordingly the assay development process seamlessly leads into an assay validation pathway,

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

2 **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.~~

both of which require fulfilment of validation criteria.. Further, more detailed guidance is provided in a series of OIE Validation Guidelines (ref.[3])that are tailored for several fundamentally different types of assay (e.g. detection of nucleic acids, antibodies, or antigens) and provide more information on specific issues related to the validation of diagnostic assays. For specific information for wildlife species, refer to the appendix to this chapter: Principles and methods for the validation of diagnostic tests for infectious diseases applicable to wildlife. *The information provided in this appendix, which is specific to wildlife species, might also be useful for domestic animal test validation, for example, where the number or availability of samples is limited.*

~~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

All laboratories should comply with the requirements of Chapters 1.1.1 (*Aquatic Manual*) or 1.1.4 (*Terrestrial Manual*) on *Quality management in veterinary testing laboratories*. This will minimise the influence of factors that do not depend on the test itself such as instrumentation, operator error, reagent choice (both chemical and biological) and calibration, reaction vessels and platforms, water quality, pH and ionicity of buffers and diluents, incubation temperatures and durations, and errors in the technical performance of the assay.

The first ~~consideration~~ step in assay development is to define the purpose of the assay, because this guides all subsequent steps in the validation process. Assay validation criteria are the characterising traits of an assay that represent decisive factors, measures or standards upon which a judgment or decision may be based. 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 such as: (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 purpose for which the assay is applied ~~analyte in question~~.

3 Note to reader: Once finalised, these guidelines will be made available on the OIE website.

Selection, collection, preparation, preservation and management of samples are critical variables in design and development of an assay to ensure valid test results. Other variables such as transport, chain of custody, tracking of samples, and the laboratory information management system are also key 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. 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 that is to be used in the assay (e.g. serum, tissue, whole blood) and representative of the species to be tested by the assay. The reference materials should appropriately represent the range of analyte concentration to be detected by the assay. Information on ~~proper~~ sample collection, preparation, preservation, management, and transport is available in chapters 1.1.1 and 1.1.2 of the *Terrestrial Manual*.

The matrix in which the targeted analyte is found (serum, faeces, tissue, etc.) may contain endogenous or exogenous inhibitors that prevent some assays from working. This is of particular concern for enzyme-dependent tests such as polymerase chain reaction (PCR) or enzyme-linked immunosorbent assay (ELISA). Other factors that affect the concentration and composition of the target analyte (particularly 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 potentially affect the ability of the assay to detect the specific targeted analyte in the sample. It is also important that biological reagents are free of extraneous agents that might otherwise lead to erroneous results.

~~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). 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~~ beginning with optimisation of the assay. After initial optimisation for an intended purpose, characteristics of the performance of the assay will be tested. The assay may need additional optimisation or may be found to be fit for purpose based on the results of the validation work.

Criteria for Assay Development and Validation

1. ~~Fitness for~~ Definition of the 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~~

13. Fitness for intended purpose(s)

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)~~ (World Organisation for Animal Health, 2008)[4] states that test methods and related procedures must be appropriate for specific diagnostic applications in order for the test results to be of relevance. In other words, the assay must be 'fit for purpose'. The qualitative and quantitative assessment of 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.c), and standardised (Section A.2.e) assay that, through accrual of validation data, provides ~~less biased and more precise estimates of DSe and DSp~~ more confidence in the assay's ability to perform according to the intended purpose. ~~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 ensure that test results provide useful diagnostic inferences about animals or populations with regard to the intended purpose ~~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 of assay development is selection of an assay type that is appropriate and that likely can be validated for a particular use (fitness for purpose).

~~a) Fitness for purpose~~

The most common purposes are to:

1) Contribute to the demonstration of 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.
3) Contribute to the eradication of disease or elimination of infection from defined populations.
4) Confirm 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~~ the OIE Validation Guidelines (ref) 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.

Further to the intended purpose, the assay needs to be defined in terms of target animal species, target pathogen(s) or condition, and sampling matrix.

4 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* and *Aquatic Manuals*.

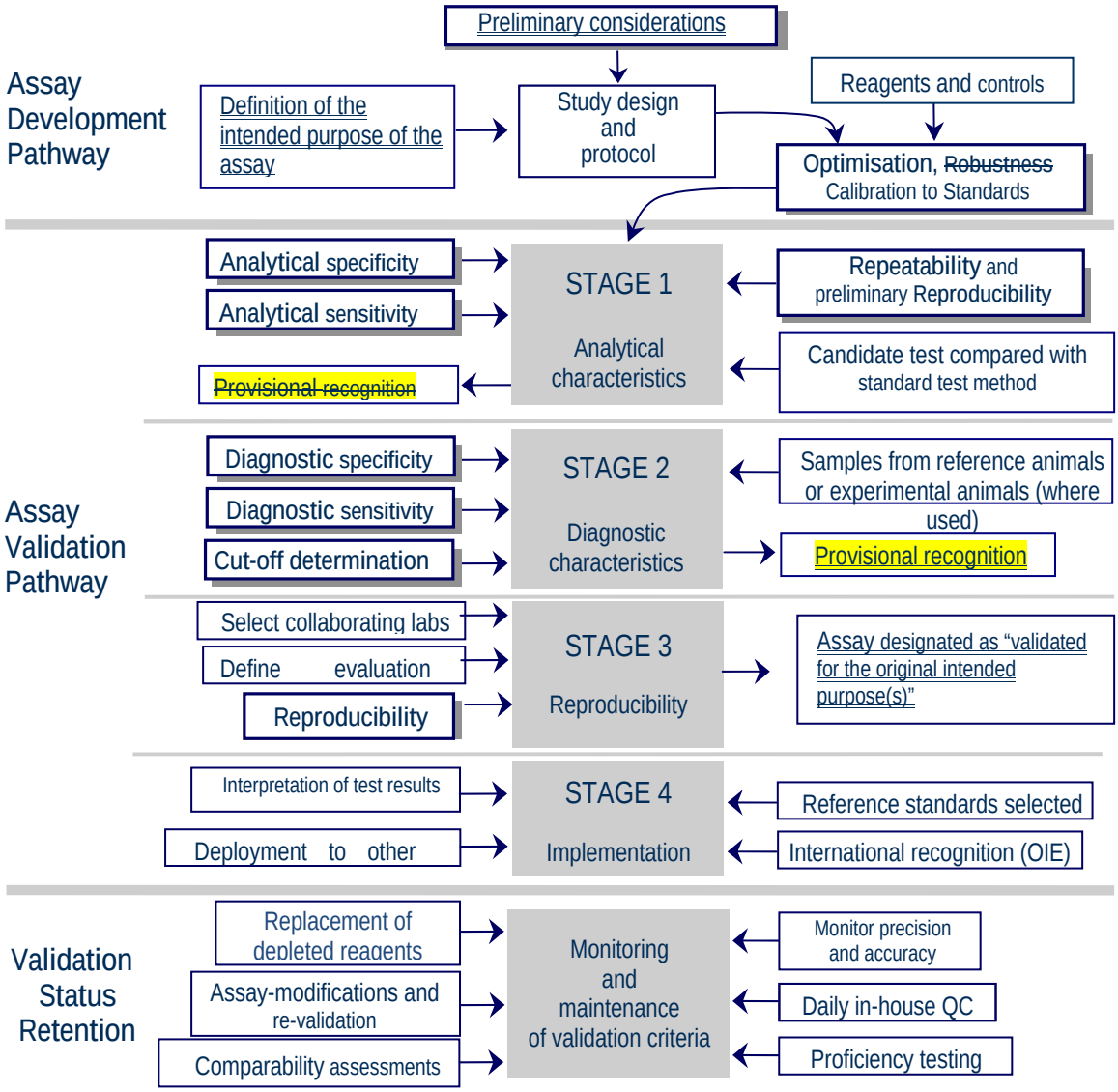

Figure 1. *The assay development and validation pathways with assay validation criteria highlighted in bold typescript within shadowed boxes.*

~~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 utility 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

~~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*.~~

a) Test method design and proof of concept

Prior knowledge, ~~Considerable~~ thought and planning need 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 the ~~Appendices~~ OIE Validation Guidelines (ref.) ~~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 any assay is dependent on analyte reference samples that reflect the target analyte, the matrix in which the analyte is found, and the population for which the assay is intended to be used. The reference samples may be sera, fluids (including meat juices) 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.

b) Operating range of the assay

The operating range of an assay is the interval of analyte concentrations or titres ~~(amounts)~~ over which the method provides suitable accuracy and precision. Accuracy is the closeness of a test value to the expected (true) value (mean or median) for a reference standard reagent of known concentration or titre. Precision[5] is the degree of dispersion (variance, standard deviation or coefficient of variation) within a series of measurements of the same sample tested under specified conditions. During development of the assay, the lower and upper ~~detection~~ limits of the operating range are determined ~~are established~~. To formally ~~establish~~ determine this range, a high ~~strong~~ positive reference sample is selected (ideally, this sample will be the same one from among the three samples described under "Optimisation" below). This high ~~strong~~ positive sample is serially diluted to extinction of the assay's response in an analyte-negative matrix of the same

5 Laboratory sources of variation that affect assay precision include: 1) within a single test run, 2) between concurrent runs, 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, categories 1–3 are estimates of repeatability, and category 4 is synonymous with reproducibility.

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. Optical Density, Cycle threshold, counts, etc.) a function of analyte concentration (amount). The curve establishes the working 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 and accuracy has been demonstrated~~. If the range is found to be unacceptable for the intended purpose, additional optimisation may be needed. ~~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.~~ The typical calibration curve for most assays is sigmoidal in shape. The data are transformed to approximate a linear relationship between response and concentration using a suitable algorithm (Findlay & Dillard, 2007).

c) Standardisation and optimisation

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. It is useful to select at least three well-defined reference samples, representing the analyte ranging from high ~~strong~~ positive to negative (e.g. high ~~strong~~, low ~~weak~~ positive and negative ~~low- and high-positive~~). These samples ideally should represent known infected and uninfected animals from the population that 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 as these samples do not truly represent the naturally-occurring matrix-agent interaction (see also OIE Validation Guideline 7). ~~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 or agent derived from culture, or diluting a high ~~strong~~ 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 the 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, distinguish the target from closely related analytes, 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.

The labour-intensive process of optimising an assay is fundamental and critical to achieving a reliable and predictable ~~quality~~ assay performance. Scientific judgment and use of best scientific practices, as provided in ~~accompanying Appendices to this chapter~~ the OIE Validation Guidelines, are ~~the best assets~~ recommended to guide optimisation of all elements of assay development and validation. The approach outlined provides a solid foundation for development of a reliable assay~~that fulfils the criteria of "robustness" and "ruggedness" when used 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~~ standardisation 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 and documented for parameters such as pH, molarity, etc. Standards for quality, purity, concentration and reactivity of biologicals 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 should be an integral part of optimisation and considered from the very beginning rather than, as is often the case ~~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, data 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.

During optimisation of an assay, it is important to take note of procedural steps and assay parameters that have a narrow range in which the assay performs optimally, as these are the critical points that ultimately affect an assay's ~~repeatability~~ reliability (see Section A.2.g). ~~In some assay types, a correct assay result is fully dependent on getting a particular step in the testing process correct, requiring~~ These changes should not produce any systematic errors (biases) in test results. For some assay types, specific steps in the procedure may have more impact than other steps on the final assay performance ~~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. Nucleic acid 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.5~~6~~ below and ~~associated Appendix 1.1.4.~~OIE Validation Guideline 6 for additional information on establishing comparability when reagents or processes 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 the *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).~~

d) 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.~~ Each different matrix to be used in an assay must be used in the validation process. Some sample matrices include inhibitory factors that interfere with the performance of specific types of assays. Serum, particularly if haemolysed, may contain factors toxic to the cells used in viral neutralisation assays, while endogenous substances found in some tissues and fluids can interfere with or inhibit ligand-binding and enzymatic-based assays ~~reactions in~~ such as 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.~~ tend to contain more interfering substances and are therefore more problematic for assay performance than are serum, blood or fresh tissues.

e) Robustness

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~~ be reproducible when transferred to other laboratories. ~~(ruggedness is addressed in Section 4).~~

The factors most likely to affect assay robustness ~~are quantitative (continuous) such as~~ include 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). Once optimisation is complete, the robustness of the assay becomes part of the assessment of repeatability. ~~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).~~

f) Calibration of the assay to standard reagents

i) *International and national ~~analyte~~ reference standards*

Ideally, OIE or other international reference standards, containing a known concentration or titre of analyte, are the reagents to which all assays are standardised (see OIE Guide 3: International Reference Antibody Standards for Antibody Assays. *In:* World Organisation for Animal Health, 2008, pp. 45–52 and also OIE Validation Guideline 7). Such standards are prepared and distributed by OIE Reference Laboratories or other international reference laboratories. National reference standards are calibrated by comparison with an international reference 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 standards ~~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~~ should be 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 (see OIE Validation Guideline 7). 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.

g) "Normalising" test results to a working standard

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 almost impossible to compare directly (semi-) quantitative data. To improve markedly 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 percent of a positive control (e.g. in an ELISA), or as the estimated 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. It is mandatory to control the (absolute) variation of the normalisation standards as otherwise normalisation can introduce a bias. ~~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.~~ OIE Validation Guidelines 1 and 3.

h) Preliminary study of the repeatability

Assessment of repeatability should begin during assay development and optimisation stages. Early knowledge of this situation elicits a critical decision point for determining whether it is worthwhile to continue with validation of the assay.

Repeatability is further verified during Stage 1 of assay validation (Section B.1.a). When the optimised test is run under routine laboratory or field conditions (Stage 4 of assay validation), repeatability is continually monitored as part of process control procedures for the duration of the life of the assay (see Section B.5.a).

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(s). 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.~~

1. Stage 1 – Analytical performance characteristics

Ideally, the design of studies outlined in the following sections should be done with assistance of a statistician and a disease expert to ensure that the sample size and experimental approach ~~provides sufficient statistical rigour~~ are valid. It is possible to design experiments that efficiently provide information on likely within- and between-laboratory sources of variation in assay precision (see footnote 3 under Section A.2.b, 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 sensitivity and 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. The number of replicates should preferably be determined in consultation with a statistician with a suggested minimum of three ~~(preferably five)~~ samples representing analyte activity within the operating range of the assay. Each of these samples is then aliquoted into the appropriate number of individual vessels as ~~three~~ identical replicates of the original sample containing the original analyte and matrix concentration (see OIE Validation Guideline 7). 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 multiple days ~~at least five separate days~~. The variation in replicate results can be expressed as standard deviations, coefficients of variation (standard deviation ÷ mean of replicates), or other possible options (see OIE Validation ~~Appendix~~ Guideline 4 on ~~measures of~~ measurement uncertainty for assessments of repeatability).

b) Analytical specificity

Analytical specificity (ASp) is the ability of the assay to distinguish the target analyte (e.g. antibody, organism or genomic sequence) from non-target analytes, including matrix components. The assessment is qualitative and the choice and sources of sample types, organisms and sequences for the ASp evaluation should reflect test purpose and assay type. See OIE Validation Guidelines 1, 2, and 3 for guidance for antibody, antigen and nucleic acid assays, respectively. ASp is documented during Stage 1 validation, and cross-reactions identified. Cross-reactivity (ASp less 100%) may be acceptable depending on the proposed use of the assay ~~for use of the test in field programmes (Stage 4) but this determination is not required in stage 1~~. The impact of cross-reactivity is further documented during Stage 2 (establishment of DSp) and assessed at Stage 4 implementation.

~~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.~~

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

The limit of detection (LOD) is a measure of the analytical sensitivity (ASe) of an assay. The LOD is the estimated amount of analyte in a specified matrix that would produce a positive result at least a specified percent of the time. Typically, estimated LOD will be based on spiking of the analyte into the target matrix. The choice of analyte(s) (e.g. species, strains) is part of the ASe definition and should be reported properly. These experiments may be designed for precise and accurate estimation of the probability point (e.g. 50% or 100%), but in some circumstances a conservative estimate of the LOD (e.g. 100%) may be acceptable. For example, in a titration using ten-fold dilutions all replicates at all dilutions might show either 100% or 0% response. There are two choices at that point. The last dilution showing 100% response may be accepted as a conservative estimate of the lower limit of detection. A more accurate estimate may be obtained by a second stage experiment using narrower intervals in the dilution scheme focusing on the region between 100% and 0%. Methods for statistical evaluation of LOD data are in the OIE Validation Guideline 5.~~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 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 test method and by an standard method.~~

d) 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, and 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.1.c., above). However, they differ from ~~routine~~ diagnostic tests because they do not require validation for diagnostic performance characteristics (Sections B.2 through B.4, below) if their results are not used to establish a final diagnosis with regard to the intended purpose. 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 in at 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[6]~~

~~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 Diagnostic Sensititivity (DSe) and Diagnostic Specificity (DSp) (see requirements for Stage 2, Diagnostic Performance, Section B.2 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 desired error margin 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 cut-off (threshold) or if no adjustment is needed, enhance confidence in the estimates of DSe and DSp.~~

~~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~~

~~6 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.~~

~~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 bBench validation of diagnostic assays can only offer provisional recognition with anticipation that full field validation will follow.~~

~~Provisional recognition of an assay by state or national authorities recognises 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. 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.~~

2. Stage 2 – Diagnostic performance of the assay

Estimates of DSe (proportion of samples from known infected reference animals that test positive in an assay) and DSp (the proportion of samples from known uninfected reference animals that test negative in an assay) 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. An estimate of the area under the receiver operating characteristic (ROC) curve ~~analysis~~ is a useful adjunct to DSe and DSp estimates for a quantitative diagnostic test because it assesses its global accuracy ~~of a quantitative diagnostic test~~ across all possible assay values (Greiner *et al.*, 2000; Zweig & Campbell, 1993). This approach is described ~~in depth~~ in ~~Appendix~~ OIE Validation Guideline 5.

~~**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. Many samples are required to achieve a high confidence (typically 95%) in the estimates of DSe and DSp when a small error margin in the estimate is desired. For example comparison of a 2~~5~~% vs 5~~2~~% error for a likely DSe or Dse of 90% and 95% confidence shows a considerable ~~reduction~~ increase (864 *vs* 138) 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 the statistically required ~~optimal number of~~ sample size will be evaluated, in which case the confidence interval calculated for DSe and DSp will indicate less diagnostic confidence in the results. ~~However, by reducing the DSe and DSp confidence levels to less than 90% usually would not be recommended.~~Sample size also may be limited by the fact that reference populations and OIE reference standards ~~"gold standards" (perfect reference standards)~~may be lacking (see ~~Appendix 1.1.4.~~ OIE Validation Guideline 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 error margin in the DSe and DSp estimates by adding more samples (of equivalent status to the original panel) 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 depending on likely value of DSe or DSp and desired error margin and ~~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. Estimated numbers are based on the normal approximation to the binomial distribution. For the number of samples required for 1%, 3%, and 4% error margin in the estimate of DSe and DSp, multiply the number of samples in the left panel of the table (2% error margin) by a factor of 4.0, 0.44, and 0.25, respectively (Dohoo *et al.*, 2010), and round the number up to next highest integer.

Percent 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% error margin 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 (for further details see OIE Validation Guideline 7).

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 or zones that have eradicated or have never had the disease in question. Such samples may be useful as long as the targeted population for the assay is sufficiently 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~~ pathogen. It may be necessary to resort to samples from animals that have been identified ~~tested~~ by another test of sufficiently high accuracy, such as a validated nucleic acid detection ~~system~~ assay. The candidate test is applied to these reference samples and results (positive and negative) are cross-classified in a 2 × 2 table. This has ~~historically~~ been called the "gold standard model" as it assumes the reference standard is perfect. A sample calculation is shown in Table 2 in section e).

~~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) Samples from animals of unknown status

When the so-called reference standard is imperfect, which is the rule with any diagnostic tests, estimates of DSe and DSp for the candidate assay based on this standard will be flawed. A way to overcome this problem is to perform a latent class analysis of the joint results of the two tests assuming neither test is perfect.

Latent-class models 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 (Branscum *et al.*, 2005; Enøe *et al.*, 2000; Georgiadis *et al.*, 2003; Hui & Walter, 1980). If a Bayesian latent class analysis is used, prior knowledge about the performance of the reference test and the candidate test can be incorporated into the analysis.

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 OIE Validation Guideline 5 for details).

~~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~~ Samples 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. Single time-point sampling of individual experimental animals can be acceptable (e.g. one sample randomly chosen from each animal). Nevertheless it should be noted that for indirect methods of analyte detection, exposure to organisms under experimental conditions, or vaccination, may elicit antibody responses that are not 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.~~ In cases when the near-impossibility of obtaining suitable reference samples from naturally exposed animals necessitates the use of samples from experimental animals for validation studies, ~~even though this has to be~~ the resulting DSe and DSp measures should be considered as less than ideal estimates of the true DSp and DSe.

d) Cut-off (threshold) determination

To obtain DSe and DSp estimates of the candidate assay, which is measured on a continuous scale, the test results first must be reduced to two (positive or negative) or three (positive, intermediate [doubtful] or negative) categories of test results. 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 cut-off(s) should reflect the intended 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.,* 1995; Greiner *et al.,* 2000; Jacobson, 1998; Zweig & Campbell, 1993 and ~~Appendix 1.1.4.~~ OIE Validation Guideline 5 on statistical considerations). If considerable overlap occurs in the distributions of test values from known infected and uninfected animals, it is ~~difficult~~ impossible to select a single cut-off that will accurately classify these animals according to their infection status. Rather than a single cut-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) (Greiner *et al.,* 1995). ~~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.1.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~~ samples

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 has selected a sample size for ~~decided that estimated~~ DSe and DSp for the new assay under the assumption that the most likely values are ~~should be~~ 97% (DSe) and 99% (DSp), respectively, with a desired confidence of 95% for both estimates. The desired error margin 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~~ from which DSe and DSp estimates ~~based on the samples tested~~ have been obtained.

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~~8% (94.0 – 98.5%)**	**92.~~9~~6%** (~~84.3~~ 85.4 – ~~96.7~~ 97.0%)**

*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% = Error margin 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~~ OIE validation guideline 5 for information on confidence limits)

In this example, the DSe estimate is as anticipated, but the DSp is much lower (92%) than the anticipated value of 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 (see OIE Validation Guideline 5 for further details).

f) Provisional assay recognition[7]

There are situations where it is not possible or desirable to fulfil Stage 2 of the Validation Pathway because appropriate samples from the target population are scarce and animals are difficult to access (such as for transboundary infectious diseases or wildlife diseases).

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. 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 desired error margin 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

7 Provisional recognition does not imply acceptance 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.

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 cut-off (threshold) or if no adjustment is needed, enhance confidence in the estimates.

Provisional recognition defines an assay that has been assessed through Stage 1 for critical assay benchmark parameters (ASe, ASp and repeatability) with, in addition, a preliminary estimate of DSp and DSe based on a small select panel of well-characterised samples containing the targeted analyte and a preliminary estimate of reproducibility. This represents partial completion of Stage 2. Preliminary reproducibility estimates of the candidate assay could be done using the select panel of well-characterised samples to enhance provisional acceptance status for the assay. The candidate test method is then duplicated in laboratories in at least two 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 the reproducibility study in Stage 3 of assay validation. In following this procedure of provisional recognition the test protocol must not be varied.

Provisional recognition of an assay by state or national authorities 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.)

3. Stage 3 – Reproducibility and augmented repeatability estimates

a) Reproducibility

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~~ preferably located in distinct or different regions or countries using the identical assay (protocol, reagents and controls). To assess the reproducibility of an assay, each of at least three laboratories should test the same panel of samples (blinded) containing a suggested minimum of 20 samples, with identical aliquots going to each laboratory (see ~~Appendix~~ OIE Validation Guideline 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~~ OIE Validation Guideline 4 for further explanation of the topic and its application).

For field tests, reproducibility should be evaluated under the conditions of intended use.

b) ~~Definition~~ Designation of a validated assay

On completion of Stage 3 validation, assuming the earlier stages have been fully and satisfactorily completed, the assay may be designated as "validated for the original intended purpose". Retention of this designation is dependent on continual monitoring of the assay performance, as described in Section 5(a).

~~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 5 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 assay 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.~~

4. Stage 4 – Programme implementation

The successful deployment of an assay provides additional and valuable evidence for its performance according to the expectations. Moreover, the (true) prevalence of the diagnostic trait in the target population is an important factor that needs to be accounted for as described below.

a) Fitness for use

~~Predictive Value (PV) of positive or negative test result. PPV 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.~~

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 utility 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. For some diseases, multiple assays might be available for use in combination in disease control and surveillance programs and hence, an assay's utility might need to be assessed by evaluating incremental changes in DSe, DSp and predictive values of the combined tests.

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).

b) Interpretation of test results

Predictive values of test results: The positive predictive value (PPV) ~~of positive or negative test result. PV+~~ is the probability that an animal that has tested positive is in fact positive with regard to the true diagnostic status. The negative predictive value (NPV) is the probability that an animal that has tested negative is in fact negative with regard to the true diagnostic status.

Predictive values of test results are an application of Bayes' theorem and are calculated as follows:

PPV = **錯誤!**

and

NPV = **錯誤!**

Where:

~~*PV+*~~*PPV* = Predictive value of a positive test result

~~*PV–*~~*NPV* = Predictive value of a negative test result

P = Prevalence of infection

DSe = Diagnostic sensitivity

DSp = Diagnostic specificity

In contrast to DSe and DSp, predictive values are influenced by the true prevalence of the true diagnostic status of ~~exposure/infection in~~ the target population. In other words, predictive values are not inherent characteristics of a specific diagnostic test, but are a function of its DSe and DSp and the local prevalence of infection in ~~the tested~~ a defined population at a given point in time.

Predictive values are of great importance to field veterinarians for the interpretation of results. For example, a PPV ~~PV+~~ of 0.9 means that an animal reacting positive to the test has 90% chance of being indeed infected and 10% probability of being a false positive.

The predictive value of a positive result also has great importance for the veterinary services in charge of the management of control or eradication programs. If we consider the inverse of the PPV ~~PV+~~ (i.e. 1/PPV ~~PV+~~) it gives the information on how much money is spent in the culling of true and false positives for each true positive animal detected by the surveillance activity. In other words, if the PPV ~~PV+~~ V is 0.67, it means that two positive animals out of three are true positives and the remaining is a false positive. Since during the application of a control program, the prevalence of infection is continually changing, the monitoring of the PPV ~~PV+~~ is a way of evaluating the costs of the program.

Furthermore, during the application of a control program it is usually advisable to change the sensitivity of the tests employed, based on the variation of prevalence of infection in the target population and on the objective of the program, the PPV ~~PV+~~ may be used to make the changes in DSe and DSp based on economic considerations. In other words, when the need for a change in DSe and DSp of the test arises, a number of putative cut-offs may be set along the ROC curve of the test validation and the relevant values of

DSe and DSp for each cut-off may be used to evaluate the expected cost for the culling of each infected animal. ~~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. 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).~~

If the purpose is establishing evidence for freedom from disease, the NPV is the more important measure. The NPV critically depends on DSe.

c) 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/.

d) 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 control or surveillance 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~~ the reproducibility should be repeated when the test is transferred from the development laboratory to the field, whether for use in local laboratories or in field 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~~ reproducibility ~~(which is mostly derived from reproducibility estimates)~~.

5. 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.~~ To retain the status of a validated assay 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 programme characterised by carefully monitoring the assay's daily performance, primarily through precision and accuracy estimates for internal controls, as well as outlier tendencies. The performance can be monitored graphically by plotting measurements from assay controls in control charts[8]. 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 on-going evaluation of the assay's performance is also essential and is usually done through assessments of precision, accuracy, and outlier~~

8 *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.

~~tendencies using control charts.~~ Reproducibility is assessed through external quality control programmes such as 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.

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 5 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.

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

Over time, modifications of the assay likely will be necessary to address changes in the intended purpose, analytes targeted (i.e. modification of the assay to adjust diagnostic performance) or technical modifications to improve assay efficiency or cost-effectiveness. For a change in intended purpose of the assay, then a revised validation from Stage 2 onwards is obligatory.

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 an infectious agent, derived from animals in different geographic locations, are known to vary ~~have different target sequences or primer sites~~ requiring revalidation of the assay for the specified target population. This is especially true for nucleic acid detection (NAD) systems as it is very common for point mutations to occur in many infectious agents (especially 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 and DSp for the assay are not compromised. Similar issues can arise with immunologically-based assays for antigen or antibody.

A similar situation may occur with ~~incursion~~ emergence of new subtypes of existing pathogens ~~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 modification to the assay affected the previously documented performance characteristics of the assay ~~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.~~ Guideline 6 for description of experiments that are appropriate for comparability testing and ~~Appendix 1.1.4.~~ OIE Validation Guideline 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). It may include changes to reagents (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 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~~ requires 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 OIE Validation Guideline 6 for a description of comparability assessment. If the comparability assessment does not suggest a ~~significant~~ change in diagnostic performance, the modified assay may be phased into routine use. If, on the other hand, differences in DSp and DSe 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 or working standard 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. 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 or samples suitable for latent class analysis. The sampling design, collection, transportation, and testing environment for the new samples should be the same as used for the original validation study. Increases in sample numbers 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) Verification of existing assays (in-house validation)

~~Text under study.~~

If a laboratory is considering the use of a validated commercial kit or a candidate assay based on published literature with validation data, some form of verification will be required to determine whether the assay complies with either the kit manufacturer's or the author's assertions, with respect to Stage 1 validation criteria, in the context of the intended application. This may require a limited verification of both ASp and ASe using available reference materials, whether they be external and/or locally acquired from the target population. Once the laboratory is confident that the assay is performing as described from an analytical perspective, then proceeding to a limited Stage 2 validation should be considered in the context of the intended application and target population before the assay is put into routine diagnostic use.

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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~~

CHAPTER 2.1.10.

NEW WORLD SCREWWORM (*COCHLIOMYIA HOMINIVORAX*) AND OLD WORLD SCREWWORM (*CHRYSOMYA BEZZIANA*)

SUMMARY

The New World screwworm[1] (*NWS*), Cochliomyia hominivorax (*Coquerel*), *and the Old World screwworm*[1] (*OWS*), Chrysomya bezziana *Villeneuve, are both obligate parasites of mammals, including humans, during their larval stages. Both species are in the subfamily Chrysomyinae of the family Calliphoridae of the order Diptera* (*true flies*). *Larvae feeding on the skin and underlying tissues of the host cause a condition known as wound or traumatic myiasis, which can be fatal. Infestations are generally acquired at sites of previous wounding, due to natural causes or to animal husbandry practices, but they may also occur in the mucous membranes of body orifices.*

Female flies are attracted to wounds, at the edges of which each female lays an average of 175 (*OWS*) *to 340* (*NWS*) *eggs. The larvae emerge within 12–24 hours and immediately begin to feed, burrowing head-downwards into the wound. After developing through three larval stages* (*instars*) *involving two moults, the larvae leave the wound and drop to the ground, into which they burrow to pupate. The duration of the life-cycle off the host is temperature dependent, being shorter at higher temperatures, and the whole cycle may be completed in less than 3 weeks in the tropics.*

Treatment is generally effected by application of organophosphorus insecticides into infested wounds, both to kill larvae and to provide a residual protection against reinfestation. Preventive measures include the spraying or dipping of susceptible livestock with organophosphorus compounds and, more recently, use of avermectins (*especially doramectin*) *as subcutaneous injections to animals 'at risk'. Strict control of the movement of animals out of affected areas also acts as a preventive measure.*

Identification of the agent*: The larvae of NWS and OWS can be easily confused with each other and with the larvae of other agents of myiasis. Accurate diagnosis involves the identification of larvae extracted from the deepest part of an infested wound. The mature, third instar*s ~~*larvae*~~ *are most reliable for this purpose, and those of NWS can be identified by their darkly pigmented dorsal tracheal trunks extending from the twelfth segment of the body forward to the tenth or ninth. This pigmentation is unique to the larvae of NWS among the species encountered in wound myiasis.* ~~*First and second instars of NWS are generally identified by the morphology of the cephalopharyngeal sclerites.*~~ *Confirmation of OWS relies on the recognition of a characteristic combination of spinulation, the number of lobes on the anterior spiracles* (*4–6*), *and pigmentation of secondary tracheae* ~~*trunks*~~*.*

In the adult stage, species in the genus Cochliomyia *can be separated from other genera involved in wound myiasis by confirmation of a metallic body colour, ranging from light blue to green,* ~~*that is usually a metallic blue/green*~~ *with three dark longitudinal stripes always present on the thorax. The separation of NWS from the very similar* C. macellaria *and the identification of adult OWS are discussed in this chapter.*

Serological tests*: At present there are no applicable serological tests, nor are they indicated in the identification of this disease. However, serology may have a future role in studies of the prevalence of myiasis.*

Requirements for vaccines and biological control*: There are no vaccines or biological products available, except for the use of sterilised male flies in the sterile insect technique* (*SIT*). *In this technique, vast numbers of sterilised male flies are sequentially released into the environment,*

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

where their matings with wild females produce infertile eggs, leading to an initial population reduction and, progressively, eradication.

A. INTRODUCTION

The New World screwworm fly (NWS), *Cochliomyia hominivorax* (Coquerel), and the Old World screwworm fly (OWS), *Chrysomya bezziana* Villeneuve, are species of two genera of the subfamily *Chrysomyinae* of the dipteran family *Calliphoridae* (blowflies). Both species are obligate parasites of mammals ~~including humans~~ and, rarely, birds. The zoonotic implications are considerable because humans, especially the young, elderly or infirm, can be infested, with severe and sometimes fatal consequences (Spradbery, 1994) even today (e.g. Khataminia *et al.*, 2011). Despite being in different genera and geographically separated, the two species have evolved in remarkable parallel. They have almost identical life histories because they fill identical parasitic niches in their respective geographical zones. The following discussion will relate to both species, except where indicated.

Unlike most other species of blowflies, adult female screwworms do not lay their eggs on carrion. Instead, they lay them at the edges of wounds on living, injured mammals or at their body orifices. Virtually any wound is attractive, whether natural (from fighting, predators, thorns, disease, and/or tick and insect bites) or man-made (from shearing, branding, castrating, de-horning, docking, and/or ear-tagging). Commonly infested natural wounds are the navels of newborn animals, and the vulval and perineal regions of their mothers, especially if traumatised. If eggs are deposited on mucous membranes, the larvae can invade undamaged natural body openings such as the nostrils and associated sinuses, the eye orbits, mouth, ears, and genitalia.

Within 12–24 hours of the eggs being laid, larvae emerge and immediately begin to feed on the wound fluids and underlying tissues, burrowing gregariously head-downwards into the wound in a characteristic screwworm fashion. As they feed, tearing the tissue with their hook-like mouthparts, the wound is enlarged and deepened, resulting in extensive tissue destruction. Infested wounds often emit a characteristic odour, which can be the first indication that at least one animal in a group is infested. Although the odour is not always apparent to humans, it is obviously highly attractive to gravid females (Hall, 1995), which lay further batches of eggs, so increasing the extent of the infestation. A severe infestation that is left untreated may result in the death of the host.

Screwworm larvae pass through three stages (or instars), separated by cuticular moults that facilitate rapid growth, and they reach maturity about 5–7 days after egg hatch. They then stop feeding and leave the wound, falling to the ground into which they burrow and pupate. The pupa develops within the puparium, a barrel-shaped protective structure formed by hardening and darkening of the cuticle of the mature larva. On completion of development, adult flies usually emerge from the puparium in the morning and work their way up to the soil surface, where they extend their wings for hardening prior to flight. Males become sexually mature and able to mate within 24 hours, but the ovaries of females need to mature over 6–7 days, and females only become responsive towards males, mating when about 3 days old. About 4 days after mating, female flies are ready to oviposit. They seek a suitable host and lay their eggs, all oriented in the same direction, like a tiled roof, firmly attached to each other and to the oviposition substrate. The numbers of eggs laid per batch vary depending on many factors (e.g. fly strain, disturbance during oviposition), but the average first batch has in the order of 175 eggs for OWS and 340 for NWS (Spradbery, 1994). Following the first egg batch, further batches are laid at intervals of 3–4 days (Thomas & Mangan, 1989). Adult flies live on average for 2–3 weeks in the field during which time they feed at flowers, and the females also take in protein, e.g. from serous fluids at animal wounds and decomposing animals.

The rate of development of the immature stages is influenced by environmental and wound temperatures, being slower at low temperatures, although true diapause does not occur. This effect is most pronounced in the off-host pupal stage, which can vary from 1 week to 2 months' duration depending on the season (Laake *et al*., 1936). Thus, the complete life cycle of NWS may take 2–3 months in cold weather ~~(Parman, 1945)~~, whereas in temperate conditions with an average air temperature of 22°C, it is completed in about 24 days (James, 1947), and in tropical conditions averaging 29°C it is completed in about 18 days (Thomas & Mangan, 1989).

The degree to which NWS and OWS can tolerate cold has had a major influence on their distributions, best documented for NWS. Historically, the range of NWS extended from the southern states of the United States of America (USA), through Mexico, Central America, the Caribbean islands and northern countries of South America to Uruguay, northern Chile and northern Argentina (James, 1947). This distribution contracted during the winter months but expanded during the summer months, producing a seasonality at its edges and year round populations in the central areas – the New World tropics. Use of the sterile insect technique (SIT) in major programmes has resulted in eradication of NWS from the USA ~~(Baumhover, 2001)~~, Mexico ~~(Graham, 1985)~~, Curacao, Puerto Rico, and the Virgin Islands and, in Central America, from Guatemala, Belize, El Salvador, Honduras, Nicaragua ~~and, in 2000,~~ Costa Rica (Wyss, 2001) and Panama. ~~The Central American eradication programme is continuing in Panama, where sterile flies were first released in July 1998. The ultimate objective is to establish a barrier zone in Panama that will become the future northern limit of NWS in the Americas.~~ Panama was recognised as free from NWS in 2006 and a permanent barrier zone was established primarily in the Darien province of eastern Panama. This serves as the northern limit of NWS in the Americas. A NWS eradication

programme was also officially launched in Jamaica in July 1998, as part of a plan to eradicate the species from the entire Caribbean. This programme ~~has~~ encountered severe setbacks due to a complex combination of management and technical difficulties~~, but is ongoing although with an uncertain future (Dyck *et al*., 2005;~~ (Vreysen *et al*., 2007), which eventually led to the failure of the programme on the island. Although NWS is a New World species, in 1988, it was detected in Libya in North Africa where it threatened to become firmly established. However, it was eradicated in 1991 by an intensive SIT campaign (~~Food and Agriculture Organization of the United Nations [FAO], 1992;~~ Lindquist *et al*., 1992). The threat of spread of screwworms aided by modern rapid transport systems is ever present, necessitating constant vigilance from quarantine and other front-line animal health and medical officers in unaffected areas. Imported cases of NWS have been reported ~~recently~~ in Mexico, USA, and even in the United Kingdom~~ (Mallon *et al*., 1999)~~.

The distribution of OWS is confined to the Old World, as the name suggests, throughout much of Africa (from Ethiopia and sub-Saharan countries to northern South Africa), the Middle East Gulf region~~ countries~~, the Indian subcontinent, and south-east Asia (from southern China [People's Rep. of] through the Malay Peninsula and the Indonesian and Philippine islands to Papua New Guinea) (James, 1947; ~~Spradbery *et al*., 1985;~~ Sutherst *et al*., 1989; Zumpt, 1965). OWS was reported from Hong Kong for the first time in 2000, infesting dogs, and a first human case was reported in 2003 (Ng *et al*., 2003). OWS myiasis has also been reported from Algeria (Abed-Benamara *et al*., 1997), in a local shepherd, ~~but~~ and in Mexico (Romero-Cabello *et al*., 2010). However, in the absence of other reported cases, particularly animal cases, a continuing presence in either region seems unlikely and the original case~~s~~ could have been ~~a~~ misidentified, emphasising the need for correct identification of samples. The situation in the Gulf area and surrounding regions is dynamic, with ~~recent~~ reports confirmed from Iran, ~~(Navidpour *et al*., 1996~~ Iraq~~ (Al-Izzi *et al*., 1999)~~ and, most recently, Yemen (Robinson *et al*., 2009). Epizootics of traumatic myiasis can follow introductions into such areas, especially where the livestock owners and veterinarians are unfamiliar with OWS (Siddig *et al*., 2005). The climatic requirements of the two screwworm species are very similar and their potential distributions, if unrestrained, would overlap considerably (Sutherst *et al*., 1989).

Organophosphorus insecticides such as dichlofenthion, fenchlorphos, and in particular, coumaphos are recommended for the treatment of wounds infested with OWS and NWS (Graham, 1979; ~~Perkins, 1987;~~ Spradbery *et al*., 1994~~ 1991~~). They have the effect of expelling the larvae, which die on the ground. To provide residual protection against reinfestation, they must be applied at 2–3-day intervals until the wound has healed. The contents of individual wound treatment sachets, e.g. 5 g of 5% coumaphos wettable powder, should be either sprinkled directly on to a wound or, more effectively, brushed into the wound as a paste after mixing with ordinary cooking oil (33 ml). Organophosphorus compounds may also be applied as aerosol sprays, in which marker dyes and bacteriostats are included, or as dusts that are puffed into the wound from plastic squeeze bottles. ~~Dichlorfenthion is used in South America as a 1% aerosol to treat NWS cases and is also effective against OWS (Perkins, 1987).~~ Any larvae that die in the wound should be removed to prevent sepsis. Close attention should always be paid to the manufacturers' safety instructions.

Direct prevention of screwworm infestation can be achieved by spraying or dipping of livestock with coumaphos (0.25% aqueous suspension of 50% wettable powder) or other organophosphorus insecticides, at the maximum concentration prescribed for external parasite control. ~~The effects of such treatment are twofold: firstly, the treatment kills larvae directly and provides residual protection; secondly, the treatment kills ticks and other external parasites, which means that there are fewer wounds available as sites for oviposition. Synthetic pyrethroids have potential for control of screwworm larvae in wounds, but there have been few reported trials of their effect on screwworms (e.g. Permethrin versus NWS; Silva *et al*., 1991).~~ Dipping or spraying of a group of animals would be indicated if any member of the group was found to be infested, or if animals were traversing or leaving an infested area, or following wound-inducing animal husbandry practices, e.g. shearing and castration. Synthetic pyrethroids have potential for control of screwworm larvae in wounds, but there have been few reported trials of their effect on screwworms.

There are few recent studies that assess insecticides for screwworm treatment and control, but many older publications describe the benefits of various macrocyclic lactones especially subcutaneous injections of doramectin, in preventing ~~A single subcutaneous injection of ivermectin (200 µg/kg) was effective against OWS in preventing navel strike of newborn calves (Perkins, 1987) and scrotal strike of castrated calves (Spradbery *et al*., 1985). Ivermectin also prevented re-strike of treated wounds of adult cattle. Cattle treated with a sustained-release bolus of ivermectin developed no OWS myiasis from 14 to 102 days after treatment (Wardhaugh *et al*., 2001). However, because of the negative effects on dung-breeding fauna, it was recommended that boluses be reserved for use in containing outbreaks of OWS. Early results suggested that ivermectin may be ineffective against NWS (Mackley & Brown, in: Graham, 1985), but more recent studies demonstrated that it can produce a significant reduction in the incidence of navel and scrotal myiasis due to NWS (Benitez-Usher *et al*., 1997; Lombardero *et al*., 1999). Although results of ivermectin trials show variation, results of doramectin trials are overwhelmingly positive (Guimaraes & Papavero, 1999). There has been an increasing number of publications reporting that a subcutaneous injection of doramectin (200 µg/kg) was up to 100% effective as a NWS prophylactic, preventing~~ infestation of ~~artificial wounds,~~ umbilical or castration wounds of calves, and infestation of post-parturient cows, for up to 12–14 days post-treatment (see in Vercruysse & Rew 2002) ~~(Anziani *et al*., 2000; Moya-Borja *et al*., 1993; Muniz *et al*., 1995). This doramectin treatment does not reduce egg-laying and, therefore, is efficient because gravid adults are not repelled and driven towards untreated animals. Effectiveness depended on factors such as cattle breed and degree of challenge. In one comparative trial, doramectin and ivermectin, both at 200 µg/kg subcutaneous injection, gave 100% and 50%~~

protection, respectively, against NWS myiasis, experimentally induced 2 hours after treatment (Moya-Borja *et al*., 1997). Doramectin also provided complete protection for 21 days and partial protection (56%) at 28 days post-treatment (Moya-Borja *et al*., 1997). In another, larger, comparative trial, doramectin had a mean efficacy of 94.6% (range 53.3 100%) compared with 43.7% (range 0 100%) for ivermectin (Caproni *et al*., 1998). Abamectin (subcutaneous injection, 200 µg/kg) gave good, but not 100%, prevention of post-castration myiasis by NWS (Anziani *et al*., 1995). Pour-on formulations of moxidectin, eprinomectin and doramectin gave poor protection against OWS myiasis (Wardhaugh *et al*., 2001) when compared with injectable formulations of doramectin against NWS. There are early indications that fipronil (a phenyl pyrazole) might be effective as a preventive of post-castration myiasis of cattle. Topical application of 10 mg/kg bodyweight of a 1% fipronil solution did not prevent oviposition by NWS, but it reduced the proportion of bulls developing active myiasis over the critical 10-day post-castration period, when most ovipositions occurred, from 65% in untreated controls to just 3% in treated animals (Lima *et al*., 2004). Similarly, topical application of an insect growth regulator (IGR), dicyclanil, to castration wounds in cattle gave good protection (>90%) against NWS myiasis (Anziani *et al*., 1998). IGRs are very specific to insects and, therefore, are less hazardous in the environment than many other groups of insecticides. Spinosad, a formulation of products derived from the fermentation of a bacterium with low mammalian and avian toxicity, was effective in treating and preventing myiasis due to NWS and OWS when applied as an aerosol spray (Snyder *et al*., 2005).

Indirect prevention of screwworm flies infestation includes the avoidance of wounding procedures at the times of year when screwworm flies are numerous, the careful handling of livestock to minimise wounding, the removal of sharp objects (e.g. wire strands) from livestock pens, and the use of measures to reduce other wound-causing parasites, in particular ticks, e.g. by dipping and by insecticide impregnated ear-tags.

To prevent the spread of the screwworms beyond their present geographical distribution limits, strict observation of the requirements for international trade, as set out in the OIE *Terrestrial Animal Health Code*, is necessary.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Identification of the eggs and first instars larvae of the agents of myiasis based on morphology is difficult, and, because these stages are relatively short lived and seldom encountered during the collection of specimens from infested wounds, they will not be considered further here.

Larvae collected for diagnosis should be removed from the deepest part of the wound to reduce the possibility of collecting non-screwworm species, which may infest the shallower parts of the wound. Living specimens should first be examined for pigmentation of the dorsal tracheal trunks (Figures 1 and 4) and then be preserved in 80% ethanol and returned to the laboratory for examination under a dissecting microscope at up to ×50 magnification (for further techniques see: FAO, 1991; Hall & Smith, 1993; Spradbery, 1991; Zumpt, 1965). If larvae are placed directly into most preservative solutions they contract and darken. However, optimal preservation of larvae, in their natural extended state, can be made by killing them in boiling water (15–30 seconds immersion) before storage in 80% ethanol. This killing method had no negative effect on subsequent extraction of mitochondrial DNA, amplified by polymerase chain reaction (PCR) (Wardhana *et al*., 2012 Hall *et al*., 2001), but it might impact other molecular techniques and this should be borne in mind.

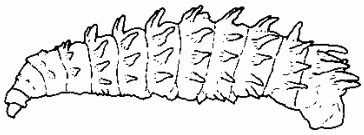
Second instar larvae: *Second instars* have only two spiracular slits in each of the posterior spiracular plates compared with the three slits of third instars (Figures 2 and 3). Second instars of NWS can be diagnosed by the presence of dark pigmentation of the dorsal tracheal trunks, for over half their length in the terminal segment. Other species have less extensive pigmentation of the dorsal tracheal trunks, for example, these trunks are pigmented for no more than one-third of their length in the twelfth segment of OWS. The anterior spiracles of second instar NWS have from seven to nine branches compared with about four branches in OWS (Kitching, 1974). More positive identification may be gained by rearing living, immature larvae to third instars. This can be done on the standard meat medium used for large-scale rearing of NWS before the introduction of gel diets, i.e. in the proportion of 1 litre water, 1.3 kg ground horse or beef meat, 50 g dried bovine blood, and 1.5 ml formalin (Taylor & Mangan, 1987), mixed and maintained at 35–38°C and 70% relative humidity. For simply rearing up larvae for identification, the exact meat and blood types are not essential, and more readily available fresh blood could be used instead of dried blood.

Third instar larvae: *Third instars* of both NWS and OWS have a robust, typical maggot shape, with a cylindrical body from 6 to17 mm long and from 1.1 to 3.6 mm in diameter, pointed at the anterior end (Laake *et al*., 1936; Spradbery, 1991). Fully mature larvae of both NWS and OWS develop a reddish-pink tinge over the creamy white colour of younger larvae. Both screwworm species have prominent rings of spines around the body and these spines appear large and conspicuous under a microscope, when compared with most non-screwworm species, the longest averaging 130 µm. In NWS the spines can be either single or double pointed, but in OWS they are always single pointed and thorn-like (Figure 2). The anterior spiracles of NWS each have from six to eleven well separated branches, but usually from seven to nine (Figure 2). In OWS, the anterior spiracles each have from three to seven branches, but usually from four to six (Figure 2). The latter character should not be used on its own

to identify OWS, because third instars of the obligate myiasis-causing species *Wohlfahrtia magnifica* (Diptera: Sarcophagidae), whose distribution overlaps that of OWS in the Middle East, have similarly branched anterior spiracles. Hence, in using any identification key, such as that in Figure 1, it is essential that each specimen be taken through the whole key to avoid misidentifications. On the posterior face of the terminal segment of both NWS and OWS, the posterior spiracular plates all have a darkly pigmented, incomplete peritreme partially enclosing three straight, slightly oval-shaped slits, which point towards the break in the peritreme. These diagnostic features are illustrated in Figure 3. Of greatest diagnostic value are the dorsal tracheal trunks, which extend forwards from the posterior spiracular plates and are darkly pigmented up to the tenth or ninth segment in NWS (Figure 1; see also: ~~FAO, 1991; 1993; Guimaraes & Papavero, 1999;~~ Hall & Smith, 1993; James, 1947; Spradbery, 1991; Zumpt, 1965 for identification keys). This feature is seen most easily in living larvae. Those in preservative may need dissection to remove opaque tissues covering the trunks. The dorsal tracheal trunks of OWS are darkly pigmented only in the twelfth segment. However, in OWS the secondary tracheae branching off the dorsal tracheal trunks are pigmented from the twelfth segment forwards to at least the tenth segment (confirmed in specimens throughout the range, from Malaysia, Bahrain and Zimbabwe; M.J.R. Hall, unpublished). Conversely, in NWS these secondary tracheae are not pigmented, only the dorsal tracheae are. Hence, the tracheal pigmentation appears almost reversed between the two screwworm species (Figure 4).

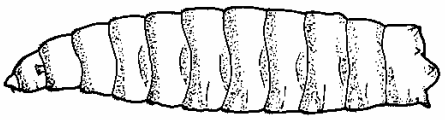
Remove larva from wound and examine gross surface structure

‘Hairy’ larva with obvious body processes

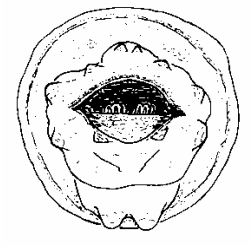


Chrysomya albiceps, C. rufifacies, C. varipes, C. villeneuvi, Fannia sp.

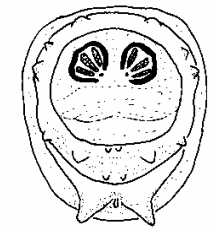
‘Smooth’ larva, with spine bands but no obvious body processes except on last segment



Posterior spiracles almost concealed in deep cavity on posterior ‘face’ of last segment



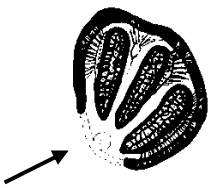
Posterior spiracles not in cavity but clearly exposed on posterior ‘face’ of last segment



Sarcophagidae

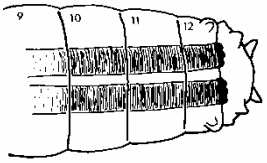
Peritreme of posterior spiracular~~e~~ plate closed

Peritreme of posterior spiracular~~e~~ plate open



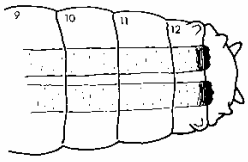
Muscidae and *Lucilia/Calliphora* species

Dorsal tracheal trunks darkly pigmented forwards from the 12th to the 10th or even 9th segment



Cochliomyia hominivorax

Dorsal tracheal trunks not darkly pigmented except possibly in posterior half of 12th segment



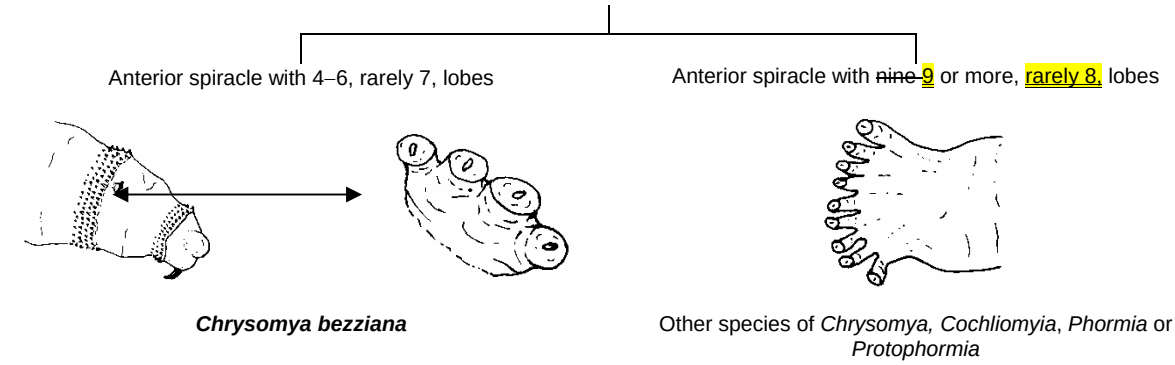


Fig. 1. *Identification key for the diagnosis of third instars ~~larvae~~ of* Cochliomyia hominivorax *and* Chrysomya bezziana *from cases of wound myiasis. To avoid misidentifications, it is essential that the key is worked through from the first step for each specimen.*

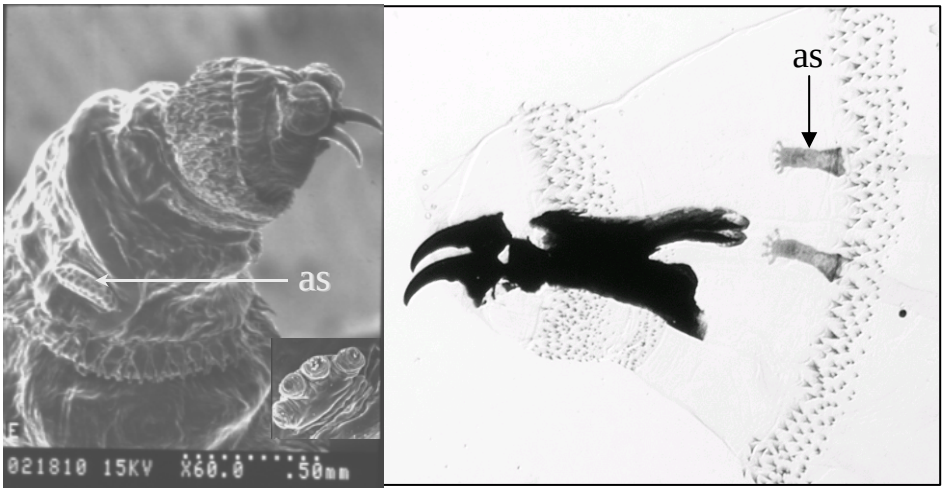


Fig. 2. *Head and ~~first~~ next two ~~thoracic~~ segments of third instar ~~larvae~~ of* Cochliomyia hominivorax (*left, viewed by scanning electron microscopy, inset is the anterior spiracle of* Chrysomya bezziana) *and of* Chrysomya bezziana (*right, viewed by compound light microscopy, note the thorn-like spines and that this slide preparation has been cleared using 10% KOH so that the anterior spiracles on both sides of the first thoracic segment are visible*); *as = anterior spiracle.*

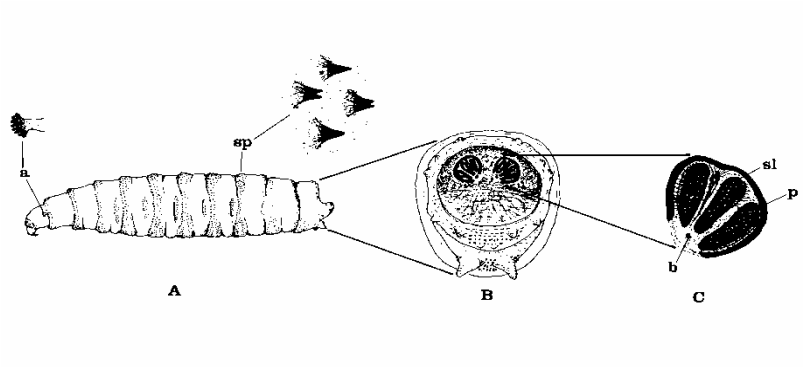


Fig. 3. *Characteristics of third instar ~~larvae~~ of* Cochliomyia hominivorax: (*A*) *whole larva, lateral aspect;* (*B*) *posterior face of terminal segment;* (*C*) *posterior spiracular plate; a = anterior spiracle; b = button adjacent to opening in peritreme; p = peritreme; sl = spiracular slit; sp = spines.* (*After Laake et al.* [*1936*].)

Adult: Adult flies needed for identification purposes are often collected using wind-oriented traps (Broce *et al*., 1977) and sticky traps (Spradbery, 1991) baited with a synthetic odour, Swormlure-4 (Mackley & Brown, 1984). A modified bucket-trap combined with a ~~and~~ newly developed attractant[2] caught an average of 3.1 times as many ~~is being developed for surveillance of~~ OWS as a sticky trap baited with Swormlure ~~in Australia~~ and was more selective for OWS (~~Rudolf~~ Urech *et al.,* 2012~~ pers. comm.~~). Real-time PCR methods can detect OWS in such bulk fly traps even when the prevalence is as low as one OWS in 1,000 other flies (Jarrett *et al*., 2010). Alternative sampling systems, using electrocuting grids or sticky surfaces at odour-baited visual targets, have been used for research purposes (Hall, 1995). Identification of adult flies is seldom required for the diagnosis of myiasis, because the larval stages are those most apparent to livestock owners and veterinary personnel. However, a brief description follows.

i) **NWS**: The body length is usually 8–10 mm ~~and has a deep blue to blue-green metallic colour~~, with three dark longitudinal stripes on the dorsal surface of the thorax. Although this fly may generally be a deep blue to blue green metallic colour, colour is variable and can range from light blue to green. This combination of colour and pattern is not shared by any other species commonly involved in wound myiasis except the secondary screwworm of the New World, *Cochliomyia macellaria* (Fabricius). These two *Cochliomyia* species can ~~also~~ be separated by the presence of black setulae on the fronto-orbital plates of the head of NWS compared with only light yellow hairs on the fronto-orbital plates of *C. macellaria*. The fifth (=fourth visible) abdominal tergite of NWS has only a very slight lateral pollinose dusting, whereas that of *C. macellaria* has a dense dusting, producing a pair of distinct, lateral, silvery-white spots. In addition, females of NWS have a dark brown-black basicosta, whereas those of *C. macellaria* have a yellow basicosta (Figure 5; see also: Dear, 1985; ~~FAO, 1993;~~ Laake *et al*., 1936; Spradbery, 1991).

ii) **OWS**: The body is up to 10 mm long and has a metallic blue, bluish-purple or blue-green colour, i.e. it is very similar to NWS, but without the thoracic stripes. The lower squama (s in Figure 5) also differs from NWS, being distinctly covered with fine hairs over its entire upper surface in OWS and other *Chrysomya* species, whereas in NWS it is hairless above, except near the base. Adults of OWS can be distinguished from other *Chrysomya* found in cases of myiasis by the combination of black-brown to dark-orange-coloured anterior thoracic spiracles (rather than pale yellow, creamy, or white), with waxy-white, lower squamae (rather than blackish-brown to dirty-grey) (Spradbery, 1991; Zumpt, 1965).

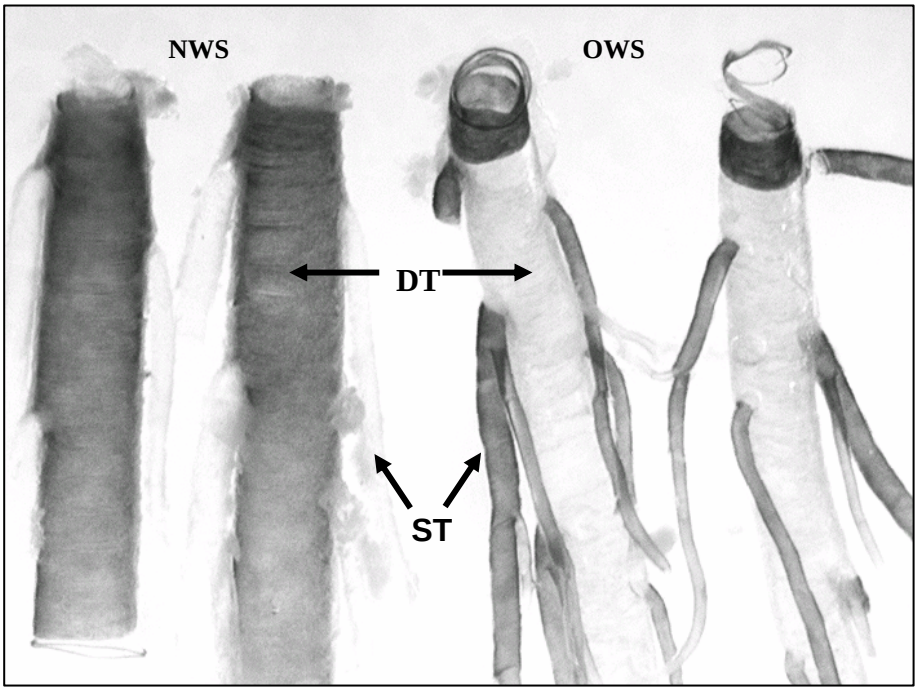


Fig. 4. *Dorsal tracheal trunks of third instar ~~larvae~~ of* Cochliomyia hominivorax *(left) and* Chrysomya bezziana *(right) dissected forwards from the posterior spiracles (top) to ninth abdominal segment (bottom). Note that the pigmentation of the main dorsal trunks (DT) and the smaller secondary ~~trunks~~ tracheae (ST) is almost reversed between the species.*

2 LuciTrap® (Bioglobal Pty Ltd) with Bezzilure-2

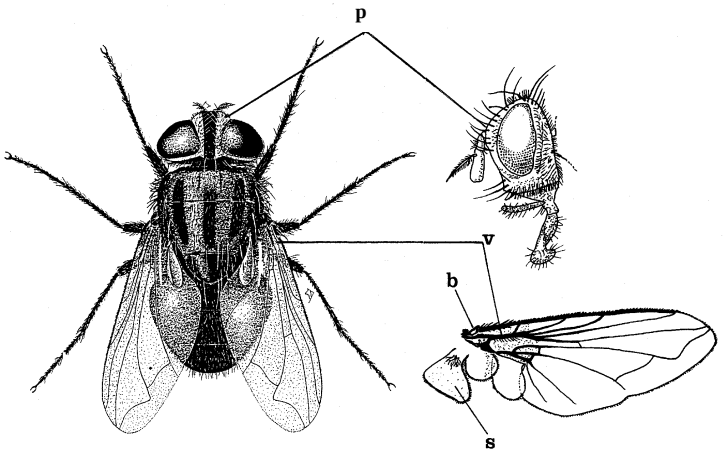


Fig. 5*. Characteristics of adult* Cochliomyia hominivorax*; note longitudinal thoracic stripes; b = basicosta; p = fronto-orbital plate, indicated from above on whole* Cochliomyia hominivorax *and laterally on head of typical calliphorid fly; s = lower squama, surface hairless except at base; v = stem vein with hairs on dorsal posterior surface.*

In addition to the standard morphological techniques discussed previously, more recent techniques for identification of screwworms and their geographical origins include cuticular hydrocarbon analysis (see in Spradbery, 1991 ~~Brown *et al*., 1998~~), and analysis of mitochondrial DNA (~~Hall *et al*., 2001; Litjens *et al*., 2001; Taylor *et al*., 1996~~ Fresia *et al*., 2011; Wardhana *et al*., 2012)~~, the complete 16,022 base pair sequence of which is known for NWS, and use of random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) (Skoda *et al*., 2002)~~. Problems with identification of larvae or adults from cases of myiasis can be referred to the OIE Reference Laboratory for New World screwworm or the FAO Collaborating Centre on Myiasis-Causing Insects and Their Identification[3].

2. Serological tests

No standardised serological tests are presently available, nor are they indicated for diagnosis of this disease. However, experimental studies have shown that serological techniques have potential value in future investigations of the prevalence of screwworm infestations in animal populations to detect antibodies to screwworm post-infestation (Thomas & Pruett, 1992).

C. REQUIREMENTS FOR VACCINES AND ~~DIAGNOSTIC~~ BIOLOGICAL CONTROL

There are no biological products such as vaccines, available currently. However, research towards development of potential vaccines is being conducted (Sukarsih Partoutomo *et al*., 2000). The only proven method of eradication of NWS relies on a biological technique, the sterile insect technique, SIT (~~Graham, 1985;~~ Lindquist *et al*., 1992), which has also been applied experimentally to OWS (Spradbery, 1994 ~~*et al*., 1989~~). In this technique, male flies sterilised in their late pupal stage by gamma or x-ray irradiation are sequentially released into the wild in vast numbers. All of their matings with wild females result in infertile eggs only, leading to a progressive population reduction and, eventually, eradication. In operational situations, SIT is supported by the insecticide treatment of screwworm-infested wounds in livestock, by strict control of livestock movement, by the quarantining of infested animals and by an active publicity campaign. SIT is very expensive because of the cost of continuous production and aerial dispersion of sterile flies. Historically, it has been considered cost effective only when used as an eradication strategy in situations where the geography would favour such a programme (e.g. ~~FAO, 1992;~~ Lindquist *et al*., 1992). ~~For many years~~ Presently, there is only one production facility for sterile adults of New World ~~sterile~~ screwworm ~~production facility~~, located ~~at Tuxtla Gutiérrez in the south of Mexico. However, a second~~

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facility opened in Pacora, Panama[4] in late 2006. An experimental facility to produce sterile OWS opened in Malaysia in 1998[5].

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NB: There is an OIE Reference Laboratory for New World screwworm (*Cochliomyia hominivorax*) (see Table in Part 4 of this *Terrestrial Manual* or consult the OIE web site for the most up-to-date list: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/ http://www.oie.int/). Please contact the OIE Reference Laboratories for any further information on New World screwworm (*Cochliomyia hominivorax*)

CHAPTER 2.1.13.

RABIES

***

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

1. General background

The prevention and control of rabies is usually a national responsibility and, in many countries, the vaccine may be used only under the control of the Competent Authority. Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements. Varying requirements relating to quality, safety and efficacy apply in particular countries or regions for manufacturers to obtain an authorisation or licence for a veterinary vaccine. Where possible, manufacturers should seek to obtain such a licence or authorisation for their rabies vaccines as independent verification of the quality of their product.

Virulent rabies virus may be used to produce inactivated rabies vaccine; consequently, the rabies vaccine production facility should operate under the appropriate biosafety procedures and practices. The facility should meet the requirements for containment outlined in Chapter 1.1.3 *Biosafety and biosecurity in the veterinary diagnostic microbiology laboratory and animal facilities* and WHO (2005).

Rabies vaccines are defined as a standardised formulation containing defined amounts of immunogens. These immunogens are either inactivated (killed), live-attenuated or biotechnology-derived as described in chapter 1.1.6.

Licensed vaccines for the parenteral vaccination of domestic animals and oral vaccines for the immunisation of wild animals are available. These vaccines are frequently used off-label in captive and free-ranging wild animals. Oral vaccines for dogs are in the experimental stage (WHO, 2007).

Lyssaviruses are classified into different viral species as described in Section A of this chapter. All rabies vaccines are produced from rabies virus (Type species RABV).

Most rabies vaccines are prepared from Pasteur's original 1882 ~~1885~~ strain and its derivative strains (Pasteur Virus, Challenge Virus Standard [CVS], Pitman-Moore~~, etc.~~), and strains isolated in the 20th century ~~more recently~~ (Flury, low egg passage [LEP], high egg passage [HEP], Street-Alabama-Dufferin [SAD], Vnukovo ~~and Kelev, etc.~~) (Geue *et al.*, 2008; Tao *et al.*, 2010; Wu *et al.*, 2011). Rabies vaccines produced in compliance with OIE requirements protect against all variants of rabies virus ~~strains of genotype 1 isolated so far~~ including other phylogroup 1 lyssaviruses (Brookes *et al.*, 2005).

Table 1. *Current rabies viruses used for challenge or for vaccine manufacturing (Bankovski et al., 2008; Metlin et al., 2008; Müller et al, 2009; Pastoret et al., 1997)*

Pasteur strain	Street Alabama Dufferin	Flury strain	Other strains
1882 France from a rabid cow ~~contaminated~~ infected by a dog	1935 USA from a dog	1939 USA from Miss Flury ~~contaminated~~ transmitted by a dog	~~Kelev: Israel from a dog (discontinued)~~
Passages in rabbits and mice then passages in cells at different levels: Pasteur virus (PV-12) Kissling (CVS-11) CVS challenge virus strain (CVS-27 Pitman-Moore (PM)	Primary cells of hamsters & pigs (10 passages) = ERA virus BHK 21 cell line passages: ~~SAD Vnukovo (USSR Russia)~~ ~~SAD Vnukovo 32~~ SAD Bern (Switzerland) SAD-B19 ~~SAG~~	136 passages in 1 day-old-chicks 40/50 passages in embryonated eggs: low egg passage (LEP) 220/227 passages in embryonated eggs: high egg passage (HEP)	CTN: China from a dog (1956)

RV-97	SAG2 ERA 333		

Conventional Rabies virus vaccines may not provide adequate cross-protection against other all lyssaviruses. especially in phylogroup II. For example, there is no protection provided against phylogroups 2 and 3, which include Mokola virus (Von Teichman *et al*., 1998), Lagos bat virus (Markotter *et al.*, 2008), and the recently identified West Caucasian bat virus (Hanlon *et al.,* 2005) and Shimoni bat virus (Kuzmin *et al.*, 2010).

Rabies is not a candidate for global eradication (Rupprecht *et al.*, 2008). From a global public health perspective however, the dog should be considered a main target for rabies elimination as it is the principal reservoir. The disease can also be successfully controlled in certain wild carnivores, such as red foxes and raccoon dogs (Cliquet *et al.*, 2012 & Aubert, 2004). Apart from dogs, other companion animals (such as cats, ferrets, etc.) and livestock pose a risk for human exposure and should be would benefit from inclusion in any national vaccination programme. Additionally, vaccination of livestock is recommended as it secures livelihoods in many parts of the world.

Rabies vaccines are formulated for their specific purpose and application either by the injectable or oral route for the immunisation of domestic animals or wildlife.

Rabies vaccines are either produced in eggs or cell culture. Nerve-tissue vaccines prepared in animals are no longer considered safe or effective for use in humans (WHO, 2005) and their use should be discontinued in animals.

Cross-neutralisation using conventional rabies virus vaccines has been demonstrated against two phylogroup I viruses: EBLV type 1 and EVLV type 2 (Brookes *et al.*, 2005).

2. Rabies vaccine for injectable use

2.1. Outline of production and minimum requirements for conventional vaccines Background

The principal rationale for the use of injectable rabies vaccine is to protect human health through the prevention and control of rabies in animals, particularly dogs. Injection ensures that the immunogen is delivered to the target species. Vaccination is also used to protect endangered species and livestock.

Live-attenuated vaccines of Flury strain (LEP or HEP) or SAD origin, have been widely used for injection in domestic animals. However, several of these products have been documented to cause rabies in vaccinated animals, and injectable use should be discontinued (Bellinger *et al.*, 1983; Esh *et al.*, 1982; Fehlner-Gardiner *et al.*, 2008).

The rabies virus glycoprotein biotechnology-derived vector vaccines are not live rabies virus vaccines (WHO, 1996). They are prepared by inserting non-infectious rabies virus nucleic acid coding for rabies virus glycoprotein into a vector such as avipox for injectable vaccine. As these do not contain live rabies virus, animals vaccinated with rabies virus glycoprotein vaccines should not be restricted from entry into countries that have restrictions on entry of animals vaccinated with live rabies virus vaccines (Taylor *et al.*, 1991). However, the ERA 333 strain used in Eastern Europe is a live rabies virus vaccine expressing a manipulated rabies virus glycoprotein.

The principles governing the preparation of inactivated rabies vaccines are identical whether they are to be used in humans or animals, although an adjuvant may be added to vaccines for animal use.

Recombinant vaccine (e.g. vaccinia rabies glycoprotein recombinant) has also proved to be effective (Brochier *et al.*, 1991; Kieny, 1984). The rabies glycoprotein recombinant vaccines are not live rabies vaccines. They are prepared by inserting non-infectious rabies nucleic acid into a vector such as vaccinia or canary pox. Since these do not contain live rabies virus, animals vaccinated with rabies glycoprotein recombinant vaccines should not be restricted from entry into countries that have restrictions on entry of animals vaccinated with live rabies vaccines.

For animals, live and recombinant vaccines are effective by the oral route and can be distributed in baits in order to immunise wild (or domestic) animals.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements.

~~Different standards apply to vaccines containing live virus modified by passage in eggs or cell cultures to reduce its virulence for the target animal, and to vaccines prepared from inactivated virus. Both types of vaccine have their advantages and disadvantages (Baer, 1991), but they can both be used to immunise animals for periods of between 1 and 3 years. Live attenuated rabies vaccines are not accepted in some countries. They are not to be relied on to protect previously unvaccinated animals that have been exposed to infection (Blancou *et al.*, 1991). Only in humans has the efficiency of post-exposure prophylaxis with vaccine alone been proven and even in these cases there is an additional strong recommendation to administer anti-rabies immunoglobulin.~~

~~All handling of the virus during manufacture and testing of vaccines must conform to the strict safety precautions specified by WHO (WHO Expert Committee on Rabies, 1992; WHO, 1996), the OIE (Chapter 1.1.2) and to national guidelines and regulations.~~

~~1. Seed management~~

2.2. Outline of production and minimum requirements for conventional vaccines

a) Characteristics of the seed

i) *Biological characteristics*

Any rabies virus strain considered for vaccine production should protect against any rabies virus variant of phylogroup 1. Selection of master seed viruses (MSVs) should ideally be based on the ease of growth in culture, virus yield, stability and antigenic spectrum (Wu *et al.,* 2011) OIE Reference Laboratories for Rabies ~~are~~ may be able to provide virus isolates as possible candidates for vaccine development ~~characterise and, in accordance with their Terms of Reference, to provide MSVs for vaccine production under governance of national legislation~~. A record of the source of the MSV should be maintained.

Biotechnology-derived vaccines are prepared in appropriate non-tumorigenic cell lines using a vector expressing the rabies virus glycoprotein.

ii) *Quality criteria*

Only MSV that has been established as pure (free from extraneous agents) and immunogenic shall be used for preparing the seed virus for vaccine production. If it is to be used as a live attenuated vaccine, the MSV must be shown not to cause clinical rabies in target animals when injected.

~~Any strain belonging to serotype 1, which has been proved to protect against field rabies viruses (currently found in the country where the vaccine is to be used), is suitable. The strain of virus used should have well known biological (e.g. pathogenicity) and antigenic properties (typing by MAbs). If it is to be used as a live vaccine, the master seed virus must be shown not to cause clinical rabies. At least two animals (preferably five to six per group) of each of the species for which the vaccine is intended and, so far as possible, any species that might be in contact with vaccine or vaccinated animals, should be tested. This can be done by inoculating in or adjacent to a major nerve, a dose equivalent to ten times the intended viral titre in one dose of the proposed final product. Animals should be observed for at least 90 days for any adverse effect attributable to the master seed.~~

~~b) Method of culture~~

~~A master cell stock of the seed virus should be prepared and kept at or below –70°C. Subculture from this stock will be used for vaccine production. Virus multiplication is verified by titration during growth of the seed virus.~~

iii) *Validation as a vaccine strain*

MSVs must be well characterised and proven to be pure and free from all extraneous agents in accordance with chapter 1.1.7 and those listed by the appropriate licensing authorities.

~~Before a vaccine is licensed, evidence of efficacy should be established by the challenge of vaccinated and control animals of each target species. The challenge should be performed at the end of the period after vaccination for which the manufacturer claims maintenance of immunity. Antibody kinetics should also be determined in order to establish the correlation between antibody titre and resistance to challenge.~~

The efficacy of the ~~produced~~ resultant vaccine is assessed by studies on every target species previously vaccinated as recommended in chapter 1.1.6 and Section C.2.1.c.iii of this chapter. ~~Protection at the end of the period of immunity is monitored by a measurement of specific neutralising antibodies and by challenge with rabies virus. The experimental conditions of this challenge should~~

~~mimic the natural conditions of infection. The challenge virus should preferably be prepared from locally isolated strains. In animals vaccinated with inactivated vaccines, the percentage of seroconversion and the mean level of antibody allow a good prognosis for survival to challenge (Aubert, 1992).~~

~~The correlation between potency in the target species and antigenic value as estimated in mice should be established (see Section C.4.c below).~~

~~For the purposes of licensing a vaccine, safety tests should be conducted in the target species. In the case of live virus vaccines used in oral vaccination campaigns (including recombinant vaccines), safety tests should also be carried out on those other species that live in the area of vaccination and could become exposed to the vaccine (Baer, 1991).~~

~~Vaccine stability is ascertained by testing batches after prolonged storage, usually 1–2 years. A process of accelerated ageing, by storage at 37°C for 1 week, is sometimes used. The storage life claimed by the manufacturer is checked by the national licensing authority. In general, it is 12–18 months for fluid vaccines, and possibly 24 months for lyophilised vaccines.~~

b) Method of manufacture

~~Whatever method is adopted, close attention should be paid to the quality of the substrate. Eggs should be of SPF origin, and the cell cultures, such as BHK cell lines, should conform to international standards of sterility and innocuity.~~

~~During manufacture, the multiplication of the virus in one of the substrates mentioned above is monitored, followed by harvesting at the most appropriate time, usually 4–6 days after inoculation of eggs or cell cultures. The virus harvest is suspended in a buffer solution at a dilution that will provide an optimum antigenicity of the end-product. If required, the suspension is either inactivated or lyophilised. An adjuvant is recommended for vaccines prepared from inactivated virus, as well as for other vaccine antigens that may be incorporated in polyvalent vaccines.~~

i) Procedures

In cell culture

The virus is used to infect a suspension or monolayers of an established cell line. Such cell culture should be proven to be free from contaminating microorganisms (see chapter 1.1.6).

Cultures are infected with cell-culture-adapted strains of ~~rabies~~ virus and incubated at the appropriate temperature for a defined period. As rabies virus does not normally cause cytopathic effect, this allows several harvests from the same culture. This material is processed and used to formulate vaccine. ~~35–36°C. These may then be used as live virus vaccines (as in Flury and SAD vaccines), or as inactivated vaccines after the addition of phenol (Semple vaccine) or some other chemical, such as beta-propiolactone.~~

~~Cell culture can also be used to grow the vector viruses (e.g. vaccinia virus) harbouring the gene coding for the expression of rabies virus glycoprotein (Kieny, 1984).~~

For inactivated (killed) vaccine the virus is inactivated by addition of an inactivant of the first order, usually β-propiolactone (BPL) or ethyleneimine (EI) in the form of binary ethyleneimine (BEI). It is important that the necessary safety precautions for working with inactivants are fully observed. Other inactivants, such as formalin or phenic acid, should not be used. The inactivant is added to a virus suspension to achieve a predetermined final concentration. Inactivation must be duly validated and documented to show the inactivation kinetics and the results of the inactivation controls. The time period for inactivant treatment and temperature used for inactivation must be validated for the actual conditions and equipment used during industrial production.

Inactivated rabies vaccines are usually formulated as liquid or freeze dried. The liquid vaccine, which is most commonly used, is prepared by adsorbing the antigen onto the adjuvant, for example aluminium hydroxide gel.

ii) *Requirements for media and substrates*

The final blend may include antifoam, phenol red dye (if permitted by the country requiring vaccine), lactalbumin hydrolysate, tryptose phosphate broth, amino acids, vitamins and buffer salts. Saponin or other polysaccharides as adjuvant could be incorporated in rabies vaccines for animals ~~ruminants~~. Addition of preservatives is recommended for multi-dose vials. The freeze-dried vaccines should be reconstituted before injection with the appropriate solvent.

In cells

The cell lines used for the production of rabies virus vaccines should be in accordance with chapter 1.1.6.

In embryonated eggs

This method of culture is used for the production of live-attenuated vaccine that contains the Flury LEP or the HEP variant strain. Their use should be discontinued as indicated in Section 2.1 above (Tao *et al.*, 2010; Wachendörfer *et al.*, 1982).

~~A modified egg adapted strain of virus is inoculated into SPF embryonated chicken eggs, which are then incubated at 38°C for 5–6 days. The virus is harvested in the form of infective embryo tissues, and is usually lyophilised and used as a live vaccine. Examples of such vaccines include those that contain the Flury low egg passage (LEP), or the more desirable high egg passage (HEP) variant strain, which is safer for some animal species such as the cat.~~

~~*In animals*~~

~~Nervous tissue vaccines prepared in animals are no longer considered safe or effective, and their use should be discontinued.~~

iii) *In-process control*

During the production process, tests are undertaken at different times before constitution of the final blend, which allows the consistency of production to be verified as in accordance with chapter 1.1.6. Tests for infectivity, sterility and inactivation are fundamental in-process controls. The formulation of the final product can be standardised using additional tests to measure viral integrity after storage, antigenic mass and glycoprotein content.

Inactivation test ~~*or innocuity test*~~

Inactivation is verified using a test for residual live virus. For this, the inactivated harvest is inoculated into the same type of cell culture as that used in the production of the vaccine or a cell culture shown to be at least as sensitive. The quantity of inactivated virus harvest used is equivalent to not less than 25 doses of the vaccine. After incubation for 4 days, a subculture is made using trypsinised cells; after incubation for a further 4 days, the cultures are examined for residual live-rabies virus by the immunofluorescence test. The inactivated virus harvest complies if no live virus is detected (~~Proceedings of the International Workshop on Alternative Methods for Human and Veterinary Rabies Vaccine Testing: State of the Science and Planning the Way Forward, which was held in Ames, Iowa, USA, October 2011 [in preparation]~~ European Pharmacopoeia, 2012b).

~~This consists of monitoring virus growth to provide an optimum titre and ensure the absence of undesirable microbial contamination.~~

~~In live virus vaccines, kinetics of virus growth should be established in order to ensure a final titre of virus correlated to the desired protection in target species.~~

~~In inactivated virus vaccines, immunogenic properties of the final product may be evaluated by *in-vitro* techniques (e.g. ELISA, agar gel immunodiffusion, antibody binding tests or infected cell staining). These evaluations will indicate the best time for harvesting the virus in cell cultures.~~

~~4. Batch control~~

iv) *Final product batch/serial tests*

After combining all of the ingredients the final blend contains the definite vaccine formulation. Filling of the final blend into vials is the last step in the production process for a batch/serial. This final batch/serial undergoes the tests described below.

Sterility

Tests for sterility and freedom from contamination of biological materials may be found in chapter 1.1.7.

Safety

Unless consistent safety of the product is demonstrated and approved in the registration dossier, and the production process is approved for consistency in accordance with the standard requirements referred to in chapter 1.1.6, batch safety testing is to be performed.

This final product batch/serial safety test is conducted to detect any abnormal local or systemic adverse reactions. For the purposes of batch/serial release, each of at least two healthy seronegative target animals is inoculated by the recommended route a minimum of a double dose of the vaccine.

The animals are observed at least daily for 14 days. The vaccine complies with the test if no animal shows adverse reactions or dies of causes attributable to the vaccine (European Pharmacopoeia, 2012b).

~~Safety tests for batches of inactivated virus vaccines are carried out by inoculation of cell culture or intracerebrally into mice to detect viable virus. A suitable safety test for live rabies vaccines should be carried out on each lot of vaccine, in the intended host species. At least three, preferably five to six animals of the intended host species should be given a dose equivalent to ten times the recommended field dose, by the recommended route of administration. The animals should be observed for 180 days for adverse reactions attributable to the vaccine (Council of Europe, 2005).~~

Residual live virus

The test is carried out using a pool of the contents of five containers.

For vaccines that do not contain an adjuvant, a suitable amplification test for residual live virus is carried out using the same type of cell culture as that used in the production of the vaccine or a cell culture shown to be at least as sensitive. The vaccine complies with the test if no live virus is detected.

For vaccines that contain an adjuvant, 0.03 ml of a pool of at least five times the smallest stated dose is injected intracerebrally into each of no fewer than ten mice, each weighing 11–15 g. To avoid interference of any microbial preservative or the adjuvant, the vaccine may be diluted more than 10 times before injection. In this case, or if the vaccine strain is pathogenic only for suckling mice, the test is carried out on 1- to 4-day-old mice. The animals are observed for 21 days. If more than two animals die during the first 48 hours, the test is repeated. The vaccine complies with the test if, from the day 3 to day 21 post-injection, the animals show no signs of rabies and immunofluorescence test carried out on the brains of the animals show no indication of the presence of rabies virus.

Batch/serial potency~~/biological activity~~

For live attenuated and current biotechnology-derived vaccines, virus titrations are reliable indicators of vaccine potency once a relationship has been established between the level of protection conferred by the vaccine in the target species and titres of the modified live vaccine. Virus titration should be carried out using cell cultures. This allows laboratories to act in accordance with the provisions of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes.

~~The amount of virus present in live attenuated and recombinant vaccines is determined by titration. Once a correlation has been established between the activity of the vaccine in the target species and virus titres, virus titrations become reliable indicators of vaccine efficacy. This is carried out using cell cultures or by the intracerebral inoculation of unweaned mice (in mice it is only possible with a few attenuated viruses). Recombinant vaccines should be monitored for the expressed rabies protein until assured that expression stablilty is maintained in the manufacturing process. Titre of the vector can then be used as a reliable indicator of vaccine efficacy.~~

The potency of inactivated vaccines is tested in mice by ~~an agent identification test, e.g. antigen-capture ELISA, by the induction of rabies virus antibodies or by protection against the agent.~~

~~In the case of adjuvanted~~ For inactivated virus vaccines, ~~an agent identification test is under development (Proceedings of the International Workshop on Alternative Methods for Human and Veterinary Rabies Vaccine Testing: State of the Science and Planning the Way Forward, which was held in Ames, Iowa, USA, October 2011 [in preparation]). The current method is~~ a serological test (Krämer *et al.,* 2010), or a challenge test ~~on mice~~ (European Pharmacopoeia, 2012~~a~~b; WHO, 1996). For inactivated virus vaccines, an *in-vitro* agent identification test has been reported (Stokes *et al.,* 2012). ~~correlation between potency in the target species and antigenic value as estimated in mice provides a reliable indicator of vaccine activity. The potency of the vaccine is established in the USA by the National Institutes of Health (NIH) test. Elsewhere, the European Pharmacopoeia test is widely adopted~~.

~~Groups of at least ten mice, aged 3–4 weeks, are inoculated with single, decreasing doses of vaccine in accordance with the European Pharmacopoeia (Council of Europe, 2005), or with two doses, 1 week apart, according to the NIH test (WHO, 1996). A sufficient number of dilutions of vaccine are compared to estimate the dilution at which 50% of the mice are protected against intracerebral challenge 14 days later (Council of Europe, 2005; WHO, 1996).~~

It is not necessary to carry out the potency tests described in Section C.2.1.b.iv.a *Serological test*, and Section C.2.1.b.iv.b *Challenge test*, for each batch/serial of vaccine produced, provided that at least one of these tests has been carried out on a previous batch/serial of vaccine and this batch/serial has been demonstrated to meet the minimum potency requirements. Under these circumstances, an alternative validated method may be used to establish batch/serial potency, the criteria for acceptance

being set with reference to the batch/serial of vaccine that has given satisfactory results in either the serological test or the challenge test as described below:

a) In the serological test, the test vaccine is compared with the standard reference vaccine by measuring the amounts of neutralising anti-rabies virus-specific antibodies in mouse serum. The test vaccine passes if it induces more antibodies than the standard reference vaccine. The test should be performed as follows:

Five mice, each weighing 18–20 g are used. Each mouse is vaccinated by a subcutaneous or intramuscular route using 1/5 of the recommended dose volume. Blood samples are taken 14 days after the injection and the sera are tested individually for rabies antibodies using the FAVN ~~rapid fluorescent focus inhibition~~ test or the RFFIT (European Pharmacopoeia, 2012b).

The vaccine meets the requirement if the average or median antibody titre is equal to or higher than that obtained with a batch/serial of vaccine that gave satisfactory results in the test described in Section C.2.1.b.iv.b: *Challenge test*.

~~In the serological test, the test vaccine is compared with a standard reference vaccine by measuring the amounts of neutralising anti-rabies virus specific antibodies in mouse serum. The test vaccine meets the requirement if the antibody titre induced by the vaccine is not less than that obtained with a batch/serial of vaccine that gave satisfactory results in the test described in Section C.2.1.b.iv.b: *Challenge test*. The serological test should be performed as follows:~~

~~Five mice, each weighing 18–20 g, are used. Each mouse is vaccinated by a subcutaneous or intramuscular route using 1/5 of the recommended dose volume. Blood samples are taken 14 days after the injection and the sera are tested individually for rabies antibody using the rapid fluorescent focus inhibition test (European Pharmacopoeia, 2012b).~~

b) In the challenge test, the test vaccine is compared with the reference vaccine by measuring the protection conferred on mice. The test vaccine passes if it induces more protection than the reference vaccine.

According to the European Pharmacopoeia, the test described below uses a parallel-line model with at least 3 points for the vaccine to be examined and the reference preparation.

Selection and distribution of the test animals

Healthy female mice about 4 weeks old, preferably in a range of 18–20 g live weight and from the same stock should be used in the test. The mice should be distributed into at least ten groups of no fewer than ten mice.

Preparation of the challenge suspension

A group of mice is inoculated intracerebrally with the CVS strain of rabies virus; when the mice show signs of rabies, they are killed, the brains are removed and a homogenate of the brain tissue is prepared in a suitable diluent. Gross particulate matter is separated by centrifugation and the supernatant ~~liquid~~ is used as challenge suspension. The suspension is distributed in small volumes in ampoules that are sealed and stored at a temperature below –60°C. One ampoule of the suspension is thawed and serial dilutions are made in a suitable diluent. Each dilution is allocated to a group of mice and each mouse is injected intracerebrally with 0.025–0.03 ml of the dilution allocated to its group. The animals are observed at least daily for 14 days and the number in each group that develop signs of rabies between day 5 and day 14 is recorded. The median infectious dose (ID_{50}) of the undiluted suspension is calculated.

Determination of potency of the vaccine to be examined

At least three serial dilutions of the vaccine are prepared for examination along with three similar dilutions of the reference preparation. The dilutions are prepared such that those containing the largest quantity of vaccine may be expected to protect more than 50% of the animals into which they are injected and those containing the smallest quantities of vaccine may be expected to protect less than 50% of the animals into which they are injected.

Each dilution is allocated to a different group of mice and each mouse is injected by the intraperitoneal route with 0.5 ml of the dilution allocated to its group. A suspension of the challenge virus is prepared 14 days after the injection such that, on the basis of the preliminary titration, it contains about 50 ID_{50} in each 0.025–0.03 ml. Each vaccinated mouse is injected intracerebrally with 0.025–0.03 ml of this suspension.

Three suitable serial dilutions of the challenge suspension are prepared. The challenge suspension and the three dilutions are allocated, one to each of four groups of ten unvaccinated mice. Each mouse is injected intracerebrally with 0.025–0.03 ml of the suspension of the dilution

allocated to its group (~~Proceedings of the International Workshop on Alternative Methods for Human and Veterinary Rabies Vaccine Testing: State of the Science and Planning the Way Forward, which was held in Ames, Iowa, USA, October 2011 [in preparation]~~ Stokes *et al.*, 2012) The animals in each group are observed at least daily for 14 days. The test is invalid if more than two mice of any group succumb within the first 4 days after challenge. The number in each group that develop signs of rabies between day 5 and day 14 after challenge is recorded.

The test is invalid unless:

- for both the vaccine being examined and the reference preparation, the 50% protective dose lies between the smallest and the largest dose given to the mice;
- the titration of the challenge suspension shows that 0.03 ml of the suspension contained between 10 and 50 ID_{50};
- the confidence limits (p = 0.95) are not less than 25% and not more than 400% of the estimated potency; when this validity criterion is not met, the lower limit of the estimated potency must be at least 1 IU in the smallest prescribed dose;
- statistical analysis shows a significant slope (p = 0.95) and no significant deviations from linearity or parallelism of the dose–response curves (p = 0.99).

The vaccine meets the OIE requirement if the estimated potency is not less than 1 IU in the smallest prescribed dose.

Application of alternative end-points

Once a laboratory has established the above assay for routine use, the lethal end-point is replaced by an observation of clinical signs and the application of an end-point earlier than death to reduce animal suffering. The following is given as an example.

The progress of rabies infection in mice following intracerebral injection can be represented by five stages defined by typical clinical signs:

Stage 1: ruffled fur, hunched back;

Stage 2: slow movements, loss of alertness (circular movements may also occur);

Stage 3: shaky movements, trembling, convulsions;

Stage 4: signs of paresis or paralysis;

Stage 5: moribund state.

Mice are observed at least twice daily from day 4 after challenge. Clinical signs are recorded at each observation. Experience has shown that using stage 3 as an end-point yields assay results equivalent to those found when a lethal end-point is used. This must be verified by each laboratory by scoring a suitable number of assays using both clinical signs and the lethal end-point.

The potency test of the National Institute of Health (NIH test), as described in the US Code of Federal Regulations (9CFR), is similar to the European test, except that a second injection of vaccine is performed one week after the first injection. Reading and calculation are identical (European Pharmacopoeia, 2012a; 9CFR, 2010).

~~A WHO international standard vaccine is available (see footnote 2) for calibration of national standards, so that the results of testing for antigenicity can be expressed in IUs. The test is not valid unless:~~

~~i) For both the vaccine to be examined and the standard preparation, the PD_{50} (50% protective dose) lies between the largest and smallest doses given to the mice.~~

~~ii) The titration of the challenge virus suspension shows that 0.03 ml of the suspension contained 25 mouse intra-cranial LD_{50} (MIC LD_{50}). The challenge dose should be in the range 12–50 LD_{50} for a valid test.~~

~~iii) The confidence interval (p = 0.95) for the test should not be less than 25% and not more than 400% of the estimated potency: statistical analysis should show a significant slope and no significant deviations from linearity or parallelism of the dose–response lines.~~

~~The vaccine passes the test if the estimated potency is not less than 1 IU per dose, in the smallest prescribed dose.~~

~~A simplified test can also be used for the purpose of anticipating which vaccines are likely to be of an antigenic value ≥1 IU per dose (Aubert & Blancou, 1982). This test used as a screening test is a good way to reduce the number of mice used in vaccine potency control tests.~~

~~**d) Duration of immunity**~~

~~Duration of immunity must be established for the product licence in the target species with a defined vaccination protocol.~~

~~**e) Stability**~~

~~The proposed shelf life must be verified by appropriate tests. These experiments include biological and physico-chemical stability tests, and should be performed on a sufficient number of batches of vaccine stored under recommended conditions.~~

~~The thermostability of live virus vaccines in liquid form is generally poor. For freeze dried inactivated virus vaccines, stability is generally granted for 2 years at 4°C.~~

~~**f) Preservatives**~~

~~Inactivated virus vaccines may contain preservatives (formalin, merthiolate). The nature and quantity of these preservatives should comply with national control regulations.~~

~~5. Tests on the final product~~

c) Requirements for authorisation/licensing/registration

i) *Manufacturing process*

For registration of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Section C.2.1.a and b) should be submitted to the authorities. This information shall be provided from three consecutive vaccine batches/serials with a volume not less than 1/3 of the typical industrial batch/serial volume.

~~**a) Safety**~~

~~See Section C.4.b.~~

ii) *Safety requirements*

Safety tests for registration of inactivated injectable rabies vaccine are identical to those described in Section C.2.1.b.iv *Safety* and need to be carried out ~~for registration purposes~~ in accordance with VICH[1] Guideline 44 Section 2.1.2, as outlined here.

For vaccines that require a single life-time dose or primary vaccination series only, the primary vaccination regimen should be used. For vaccines that require a single dose or primary vaccination series followed by booster vaccination, the primary vaccination regimen and an additional dose should be used. For convenience, the recommended intervals between administrations may be shortened to an interval of at least 14 days. Evaluation of the one or repeat dose testing should be conducted using either a pilot or production batch containing the maximum release potency or, in the case where maximum release potency to be licensed is not specified, then a justified multiple of the minimum release potency should be used.

In general, eight animals per group should be used unless otherwise justified. For each target species, the most sensitive class, age and sex proposed on the label should be used. Seronegative animals should be used ~~for live vaccines~~. In cases where seronegative animals are not available, the use of alternatives should be justified.

If multiple routes and methods of administration are specified for the product concerned, administration by all routes is recommended. If one route of administration has been shown to cause the most severe effects, this single route may be selected as the only one for use in the study. Special attention shall be paid to the site of injection, especially for cats. Site recommendations should be followed.

Biotechnology-derived injectable vaccines do not shed virulent rabies virus, but other safety concerns may be evident (Roess *et al.*, 2012). Specific requirements for safety of this type of vaccine are described in chapter 1.1.6 for biotechnology-derived vaccines.

Tests for reversion to virulence of modified live vaccines (MLV) ~~and biotechnology-derived vaccines~~ should be done in accordance with chapter 1.1.6 ~~(under revision)~~.

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

Precautions and hazards

Current injectable rabies vaccines are innocuous if inactivated, not adjuvanted and present no toxic hazard to vaccinators. For adjuvanted vaccines, live attenuated vaccines and biotechnology-derived vaccines, warnings shall be provided by manufacturers that medical advice shall be sought in case of self-injection

~~**b) Potency**~~

~~See Section C.4.c.~~

iii) *Efficacy requirements*

~~Safety tests for registration of inactivated injectable rabies vaccine are identical to those described in Section C.2.1.b.iv *Safety*. Safety tests for registration of inactivated injectable rabies vaccine need to be carried out for registration purposes in accordance with VICH Guideline 44 Section 2.1.2, as outlined here.~~

In herbivores, as a minimum requirement, efficacy can be demonstrated by serology (European Pharmacopoeia, 2012a). In other species efficacy is demonstrated by challenge.

Test animals shall be uniform and have no neutralising antibodies to rabies as determined by the serum neutralisation tests that are prescribed tests for international trade (see Section B of this chapter).

For challenge tests, a challenge virus is prepared to determine the dose and route that is sufficient to induce clinical signs of rabies in at least 80% of unvaccinated control animals. As soon as clinical signs of rabies are observed, animals are killed and rabies is confirmed using the diagnostic tests described in Section B of this chapter.

For efficacy tests in vaccinated animals, such as dogs, 25 or more animals shall be used as vaccinates. The vaccine formulation used for the efficacy trial is the minimum to be used for routine production. Ten or more additional animals shall be added as controls. At the end of the period claimed for duration of immunity, vaccinates and controls are challenged with the predetermined dose as described above. Animals are observed at least daily for 90 days after challenge. As soon as clinical signs of rabies are observed, animals are killed and rabies is confirmed using appropriate diagnostic tests. At the end of the observation period, all surviving animals are killed and their brains are tested using the diagnostic tests described in Section B of this chapter.

Requirements for acceptance in challenge tests shall be death due to rabies in at least 80% of the control animals while at least 22 of 25 or 26 of 30 or a statistically equivalent number of the vaccinates remain free of rabies for a period of 90 days.

iv) *Stability*

As described in chapter 1.1.6.

v) Duration of immunity

As part of the authorisation procedure the manufacturer should be required to demonstrate the *duration of immunity* of a given vaccine by either challenge or the use of a validated alternative test, such as serology at the end of the claimed period of protection.

3. Rabies vaccines for oral use

3.1. Background

The concept of oral vaccination of rabies in animals was developed in the 1970s as a result of the work of George Baer in the USA (Baer *et al.*, 1971), continued by Franz Steck in Switzerland (Steck *et al.*, 1982). All vaccines currently used for oral vaccination programmes are either MLV or biotechnology-derived vaccines (BDVs), which are constructed by insertion of a rabies virus glycoprotein gene into a viral vector. Pox viruses (e.g. vaccinia) and adenoviruses are the vectors most commonly used. Immunisation occurs by uptake of the oral vaccine through the lymphoid tissue of the oral mucosa and tonsils, where expression of viral proteins stimulates the immune system. The majority of vaccine viruses are destroyed by the gastric environment in the stomachs of vaccinates. The accessibility of feral and wild animals for parenteral vaccination is problematic, and oral vaccination offers an effective solution. Of paramount consideration for oral vaccine use is safety, not only for the target animals, but for the environment and other species, including humans, who may come in contact with the vaccine (see chapter 1.1.6.).

The Veterinary Public Health Department of WHO was instrumental in defining the requirements for guaranteeing the safety and efficacy of oral vaccines both for the target species and non-target species (especially humans) who might be in contact with baits or a recently vaccinated animal (WHO, 1989; 2005; 2007).

3.2. Outline of production and minimum requirement for vaccines

In addition to the requirements outlined in chapter 1.1.6, the following specific requirements must be met.

a) Characteristics of the seed

The seed is a preparation of a suitable immunogenic strain of a highly attenuated rabies virus or a glycoprotein (G) vectored virus. The history of the MSV, its immunogenic properties, safety and absence of reversion to virulence shall be well characterised, including the presence of genetic markers for MLV. A full genome sequence of the seed virus should be submitted to the regulatory authority and deposited in a public database for verification of identity and genetic stability. For biotechnology-derived MSV, additional information on recombination ~~or genomic re-assortment~~ should be considered, as a theoretical risk exists for the potential of genetic transfer and exchange with other viruses.

b) Method of manufacture

i) *Procedure*

The seed virus is used to infect a suspension or monolayers of an established cell line. These cell cultures should be proven to be non-tumorigenic and free from contaminating microorganisms.

ii) *In-process control*

During the production process, tests are undertaken at different times before constitution of the final blend, which allow the consistency of production to be verified. Tests for infectivity and sterility are fundamental in process controls.

iii) *Final product batch/serial tests*

After combining all of the ingredients the final blend contains the definitive formulation that is either used in a freeze-dried or in liquid form. Filling the final blend into sachets/capsules to be included in baits or filling directly into the bait is the last step of production of a batch/serial. This final batch/serial undergoes the tests described below:

Sterility

This test may be done before or after filling the bait. Tests for sterility and freedom from contamination with biological materials are described in chapter 1.1.7.

Identity

The identity of the immunogen is tested using rabies anti-serum monospecific of the glycoprotein G for biotechnology-derived vaccine, and for MLV a test is carried out to demonstrate the presence of the genetic marker.

Batch/serial purity

For MLV, 1 in 10 and 1 in 1000 dilutions of the vaccine are inoculated into susceptible cell cultures. The dilutions are incubated at 37°C. After 2, 4 and 6 days, the cells are stained with a panel of monoclonal antibodies that do not react with the vaccine strain but that react with other strains of rabies vaccine (for example, street virus, Pasteur strain). The vaccine complies with the test if it shows no evidence of contaminating rabies virus (European Pharmacopoeia, 2012b).

Safety

Unless consistent safety of the product is demonstrated and approved in the registration dossier, and the production process is approved for consistency in accordance with the standard requirements referred to in chapter 1.1.6 of this *Terrestrial Manual*, batch safety testing is to be performed as follows: two healthy dogs are administered orally with ten doses and are observed for 14 days. In addition, a 0.5 ml dose is injected by the intraperitoneal or subcutaneous routes into eight mice, which are then observed for 1~~0~~4 days. If any adverse reactions attributable to the products occur in any animals during the observation period, the batch/serial is unsatisfactory.

Batch/serial potency

For live attenuated and biotechnology-derived vaccines, virus titrations are reliable indicators of vaccine potency once a relationship has been established between the level of protection conferred by the vaccine in the target species and titres of the vaccine. Virus titration should be carried out using cell cultures. This allows laboratories to act in accordance with the provisions of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes. The potency of a biotechnology-derived vaccine can also be determined by measuring seroconversion in vaccinated animals. More than 80% of vaccinates should develop a titre of >0.5 IU/ml.

c) Requirements for authorisation/registration/licensing

i) *Manufacturing process*

For registration of a vaccine, all relevant details concerning manufacture of the vaccine and quality control testing should be submitted to the Competent Authority. This information shall be provided from three consecutive vaccine batch/serials with a volume not less than 1/3 of the typical industrial batch/serial volume.

The in-process controls are part of the manufacturing process.

ii) *Safety requirements*

In accordance with chapter 1.1.6, safety tests are required in each species for which the product is indicated. For purposes of this class of product, only the overdose and reversion-to-virulence safety tests are required.

Tests for reversion to virulence of MLVs and safety testing of biotechnology-derived vaccines should be done in accordance with chapter 1.1.6 ~~(under revision)~~.

Modified live vaccines (MLV)

- *In target species*

For the overdose safety test, a 10 × dose of the vaccine suspension is administered, preferably using a syringe, via the oral route to ten young animals (less than 6 months of age for wild animals and less than 10 weeks for dogs), that are free of rabies antibodies. After administration, the possibility of excretion of vaccine virus in the saliva of the animals described above should be assessed by taking swabs several times within the first day after oral immunisation and then daily over 7 days. Any virus recovered should be characterised. The animals are observed for 180 days. Particular attention shall be paid to neurological signs and sudden death and shall be investigated using the prescribed tests for international trade (see Section B of this chapter). At the termination of the study, the brain should be examined for vaccine virus presence.

The test is satisfactory if no adverse reactions attributable to the vaccine are observed and if no virus is detected in the brain. Virus recovered in swabs should be the vaccine strain and be consistent temporally and quantitatively with limited viral replication.

- *In non-target species*

A representative group of species that are susceptible to rabies and likely to consume the baits should be investigated. At least ten animals of each species should be tested orally with a field dose of vaccine and observed according to their known incubation period for rabies.

As testing wild animals might prove to be difficult and should be kept at a minimum, additional tests in laboratory rodents are recommended. Rodents (i.e. mice and rats) should be tested using at least 20 animals per test, inoculated orally with the amount of vaccine strain equivalent to one maximum oral dose. The same number of contact animals should be used for investigation of virus transmission. All animals should be observed daily for at least 30 days. Animals that die from causes not attributable to rabies are eliminated.

Safety studies should also be undertaken in non-human primates.

Biotechnology-derived vaccines (BDV)

- *In target species*

For the overdose safety test, a 10× dose of the vaccine suspension is administered, preferably using a syringe, via the oral route to ten animals that are free of rabies antibodies. After administration, the possibility of excretion of vaccine virus in the saliva of these animals should be assessed by taking swabs several times within the first day after oral immunisation and then daily

within 7 days. Any virus recovered should be characterised. The animals should be observed for 14 days.

The test is satisfactory if no adverse reactions attributable to the vaccine are observed. Virus recovered from swabs should be the vaccine strain and be consistent temporally and quantitatively with limited viral replication. For vaccine intended for use in dogs, absence of the virus should be demonstrated 4 days post-immunisation.

- *In non-target species*

A representative group of species that are susceptible to the virus vector and likely to consume the baits should be investigated. At least ten animals of each species should be tested orally with a field dose of vaccine and observed according to their known incubation period for the vector used.

As testing wild animals might prove to be difficult and should be kept to a minimum, additional tests in laboratory animals susceptible to the vector are recommended. Laboratory animals should be tested using at least 20 animals per test, inoculated orally with the amount of vaccine strain equivalent to one maximum oral dose. The same number of contact animals should be used for investigation of virus transmission. All animals should be observed daily for at least 14 days. Animals that die from causes not attributable to the disease caused by the vector are eliminated.

If the viral vector is infectious for humans, safety studies should also be undertaken in non-human primates and in immunocompromised mice.

The product should also comply with the section in chapter 1.1.6 on licensing of products derived through biotechnology (under revision).

Precautions hazards

The release of oral vaccines into the environment shall comply with the requirements in chapter 1.1.6 (under revision). Current oral rabies vaccines are innocuous when presented in bait form and present no toxic hazard to vaccinators. For leaks from ruptured sachets containing vaccines, warnings shall be provided by manufacturers that medical advice shall be sought in the event of inadvertent contact, especially when contact is with mucosal membranes, skin or skin abrasions.

Prior to initiating vaccination campaigns, public health officials should be informed and public education provided, particularly not to touch baits or be in contact with animals that have recently consumed baits.

Public Health information with respect to the risk of oral vaccines in specific human population groups is provided by WHO (WHO, 2005).

iii) *Efficacy requirements*

~~**6. Oral vaccination**~~

~~The concept of oral vaccination is unique: as stray or wild animals are out of physical reach, dropping vaccine baits into their environment is the only way to immunise them. In the 1980s and 1990s, the Veterinary Public Health Department of WHO organised several meetings of rabies experts to define the requirements for guaranteeing the safety and efficacy of vaccines both for the target species (red fox, raccoon dog, skunk, dog, etc.) and nontarget species, namely wild rodents and any other wild and domestic species that might be in contact with baits or a recently vaccinated animal (WHO, 1989; WHO Expert Committee on Rabies, 2005).~~

~~Several guidelines have been established for the quality criteria that vaccines have to satisfy before marketing; the most precise documents are those produced by WHO, the European Pharmacopoeia and the European Commission (European Commission, 2002; European Directorate for the Quality of Medicines, 2007; WHO Expert Committee on Rabies, 2005). Available oral vaccines have been extensively tested by different routes (cerebral, muscular and oral) in a variety of species: puppies and adults of carnivores, avian species, nonhuman primates, rodents and immunocompromised mice. Nonhuman primates have been added to this list since the discovery in 1992 that the original SAD Bern strain is highly pathogenic for baboons by the oral route (Bingham *et al.*, 1992).~~

~~All vaccines currently used for oral vaccination programmes are either modified live-virus vaccines or live recombinant vaccines. At the present time, two oral vaccines are recommended by WHO (WHO Expert Committee on Rabies, 2005): a recombinant vaccine – VRG vaccine, and a highly attenuated vaccine – SAG2.~~

~~The production controls are closely related to the ones used for parenteral vaccines. The major differences concern three points:~~

~~i) Safety of the vaccine for man, target and non-target species.~~

~~ii) Efficacy of the protection induced by the vaccine.~~

~~iii) Monitoring of the impact of oral vaccination campaigns in the field.~~

Efficacy shall be demonstrated in each species for which the vaccine use is claimed by the manufacturer, using in a first step a direct oral instillation and the virulent virus in challenge tests.

Test animals shall be uniform and have no neutralising antibodies to rabies as determined by the serum neutralisation tests that are prescribed tests for international trade (see Section B of this chapter).

For challenge tests, a challenge virus is prepared to determine the dose and route that is sufficient to induce clinical signs of rabies in at least 80% of unvaccinated control animals for each target species. As soon as clinical signs of rabies are observed, animals are killed and rabies is confirmed using the diagnostic tests described in Section B of this chapter.

For efficacy tests in vaccinated animals, at least 25 animals shall be used as vaccinates. The titre of the vaccine virus that is used in the efficacy test establishes the minimum immunising infectious dose. The volume must not be greater than the volume to be included into the bait. Ten or more additional animals shall be added as controls. After 30 days, vaccinates and controls are challenged with the predetermined dose as described above. Animals are observed daily for 90 days after challenge. As soon as clinical signs of rabies are observed, animals are killed and rabies is confirmed using appropriate diagnostic tests. At the end of the observation period, all surviving animals are killed and their brain tissues tested using the ~~diagnostic~~ virus identification tests described in Section B of this chapter.

Requirements for acceptance in challenge tests shall be death due to rabies in at least 80% of the control animals while at least 22 of 25, 26 of 30 or a statistically equivalent number of the vaccinates remain free of rabies for a period of 90 days.

In a second step, ~~subsequent~~ efficacy studies should be conducted using oral vaccine with manufactured baits ready to be used in the field. The vaccine should have a minimal titre corresponding to at least ten times the protective dose obtained with the same vaccine experimentally by direct oral instillation (Blancou *et al.*, 1986).

The protection status cannot be checked by serology only; a virulent challenge with the appropriate challenge rabies virus is necessary. The same challenge conditions and requirements as above apply except that the challenge virus will be administered 180 days after vaccination instead of 30 days.

Once the minimum immunising dose has been established in one species, the efficacy study for additional species can be limited to a study using baits. The bait is an integral part of the product and should ideally meet certain criteria:

- Designed for and attractive to the target species and adapted to the mode of distribution. It should not be attractive to humans;
- Keep its form and shape under a wide range of temperature and weather conditions;
- Allow the incorporation of a marker, topical or systemic;
- Ingredients are non-harmful, comply with animal feed standards and should not interfere with vaccine activity;
- Feature a labelling system with a public warning and identification of the product.

iv) *Stability*

A minimum of five samples of the final product ready to be used are incubated ~~in the field~~ at 25°C for 5 days. The vaccine is titrated three times. The mean virus titre must be at least the minimum virus titre stated on the label or as approved for end of shelf life. The bait is heated at 40°C for 1 hour, and the bait casing complies with the test if it remains in its original shape and adheres to the vaccine container.

~~**a) Safety considerations**~~

~~For oral vaccination, either attenuated rabies strains or live recombinant vaccines may be used. The vaccine should not induce any adverse signs in target and nontarget species. For vaccines used for dog immunisation, saliva should be checked for the absence of vaccinal virus because of possible contact with humans.~~

The attenuated rabies virus-based vaccines must achieve the lowest residual pathogenicity for target and nontarget species (European Commission, 2002); this is of utmost importance in the case of oral vaccination of dogs as dogs are often in close contact with humans (WHO Expert Committee on Rabies, 2005).

The recombinant vaccines cannot induce any risk of rabies; the safety controls concern only the possible residual pathogenicity of the recombined parental virus.

b) Protection induced by the vaccine

The protection induced by the vaccine must be tested not only with the virus itself (to determine the minimal vaccinating dose) but also with manufactured baits ready to be used in the field. For foxes for instance, the vaccine should have a minimal titre corresponding to at least ten times the 100% protective dose (obtained with the same vaccine experimentally by direct oral instillation) (Blancou *et al.*, 1986).

The protection status cannot be then checked by serology only; a virulent challenge with the homologous street rabies virus is necessary because of the important implication of cell mediated immunity in response to oral vaccination (European Directorate for the Quality of Medicines, 2007).

c) Monitoring the impact of oral vaccination

The stability of the vaccine in the field is important. The European Commission stresses the importance of checking the 100% protective dose after 7 days of exposure at 25°C (European Commission, 2002). Each vaccine bait should be tested for stability with a melting point above 40°C, and the blister or sachet containing the vaccine should still be covered by the bait casing after 7 days exposure at 40°C (European Commission, 2002

Aerial distribution of baits is the only way to perform an homogenous, rapid and sufficient distribution for wildlife vaccination. Quality control measures should be used to monitor different key points of baiting; control of vaccine titre, control of area coverage by air and of baiting density should at least be constantly monitored. The cross border cooperation between neighbouring countries is also needed to avoid any unvaccinated area along the border.

For wildlife in Europe, two campaigns are performed yearly: the spring one aims at vaccinating the young population of the target species, its period should then be fixed according to the biology of the target species. The autumn campaign concerns both adult and young animals. It is generally admitted that four campaigns (i.e. 2 years) should be conducted after the last rabies diagnosis.

The impact of vaccination on the host/vector population is monitored in two different ways:

- Directly by measuring the bait uptake by the wild target species. This supposes that a biomarker (generally tetracycline) is included in the bait casing. The same examination allows the age of animals to be determined.

- Directly by measuring the serological response of target animals. This serological control is better done using validated ELISA techniques (Cliquet *et al.*, 2000; Servat *et al.*, 2007) as they are more robust than seroneutralisation tests when testing poor quality field specimens.

- Indirectly by measuring the incidence of rabies in the vaccinated area. Typing of field isolates should be performed (WHO Expert Committee on Rabies, 2005) either by using MAbs or by sequencing positive samples from areas where the target species have been vaccinated with attenuated vaccines to possibly distinguish vaccine and field virus strains.

The first two controls should be performed on specially killed animals to collect good quality samples. Rabies monitoring is more sensitive when performed in found dead or ill animals.

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NB: There are OIE Reference Laboratories for Rabies
(see Table in Part 4 of this *Terrestrial Manual* or consult the OIE web site for the most up-to-date list:
http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/ http://www.oie.int/).
Please contact the OIE Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for rabies

CHAPTER 2.1.20.

WEST NILE FEVER

SUMMARY

West Nile fever is a mosquito-borne viral disease which can affect birds, humans and horses causing inapparent infection, mild febrile illness, meningitis, encephalitis, or death. West Nile virus (*WNV*) *is a member of the genus* Flavivirus *in the family* Flaviviridae*. The arbovirus is maintained in nature by cycling through birds and mosquitoes; numerous avian and mosquito species support virus replication. For many avian species, WNV infection causes no overt signs while other birds, such as American crows* (Corvus brachyrhynchos) *and blue jays* (Cyanocitta cristata), often *succumb to fatal systemic illness. Among mammals, clinical disease is primarily exhibited in horses and humans.*

Clinical signs of WNV infection in horses arise from viral-induced encephalitis or encephalomyelitis. Infections are dependent on mosquito transmission and are seasonal in temperate climates, peaking in the early autumn in the Northern Hemisphere. Affected horses frequently demonstrate mild to severe ataxia. Signs can range from slight incoordination to recumbency. Some horses exhibit weakness, muscle fasciculation, and cranial nerve deficits. Fever is not a consistently recognised feature of the disease in horses.

Identification of the agent: *Bird tissues generally contain higher concentrations of virus than equine tissues. Brain and spinal cord are the preferred tissues for virus isolation from horses. In birds, kidney, heart, brain, liver or intestine can yield virus isolates. Virus can also be isolated from mosquitoes. Cell cultures ~~(using, for example, rabbit kidney or Vero cells)~~ are used most commonly for virus isolation. WNV is cytopathic in susceptible mammalian culture systems. Viral nucleic acid and viral antigens can be demonstrated in tissues of infected animals by reverse-transcription polymerase chain reaction* (*RT-PCR*) *and immuno-histochemistry, respectively. ~~The most sensitive method for identifying WNV in equine tissues is a nested format of the RT-PCR procedure.~~*

Serological tests: *Antibody can be identified in equine serum by IgM capture enzyme-linked immunosorbent assay* (*IgM capture ELISA*), *haemagglutination inhibition* (*HI*)*, IgG ELISA, plaque reduction neutralisation* (*PRN*) *or virus neutralisation* (VN)*. The ELISA, VN and PRN methods are most commonly used for identifying antibody against WNV in avian serum. In some serological assays, antibody cross-reactions with related flaviviruses, such as St Louis encephalitis virus, Usutu virus, Japanese encephalitis virus, or tick-borne encephalitis* (*TBE*) *virus may be encountered.*

Requirements for vaccines ~~and diagnostic biologicals~~: *A formalin-inactivated WNV vaccine derived from tissue culture, WNV live canarypoxvirus vectored vaccine, a WNV DNA vaccine and a chimeric vaccine are licensed for use in horses.*

A. INTRODUCTION

West Nile virus (WNV) is a zoonotic mosquito-transmitted arbovirus belonging to the genus *Flavivirus* in the family *Flaviviridae* (Smithburn *et al*., 1940). The genus *Flavivirus* also includes Japanese encephalitis virus (see chapter 2.1.7), St Louis encephalitis virus, Murray Valley virus, Usutu virus, and Kunjin virus, among others (Burke & Monath, 2001). WNV has a wide geographical range that includes portions of Europe, Asia, Africa, Australia (Kunjin virus) and in North, Central and South America. Migratory birds are thought to be primarily responsible for virus dispersal, including reintroduction of WNV from endemic areas into regions that experience sporadic outbreaks (Burke & Monath, 2001). WNV is maintained in a mosquito–bird–mosquito transmission cycle, whereas humans and horses are considered dead end hosts. Genetic analysis of WN isolates separates strains into ~~two clades~~ multiple lineages (Mackenzie *et al.*, 2009; Vazquez *et al.*, 2010). Lineage 1 isolates are found in northern

and central Africa, Israel, Europe, India, Australia (Kunjin virus) and in North and Central America, and Columbia and Argentina in South America (Morales *et al*., 2006). Lineage 2 strains are endemic in central and southern Africa and Madagascar, with co-circulation of both virus lineages in central Africa (Berthet *et al*., 1997; Burt *et al*., 2002). ~~There has been a recent report of linage 2 from Hungary.~~ Circulation of WNV strains of lineage 2 have recently been reported in Hungary (Bakonyi *et al.*, 2006), Austria (Wodak *et al.*, 2011), Russia (Platonov *et al.*, 2011), Romania (Sirbu *et al.*, 2011), Greece (Danis *et al.*, 2011) and Italy (Savini *et al.*, 2012). While recent human and equine outbreaks have been due to lineage 1 or 2 viruses, strains from each lineage ~~have been~~ might be implicated in human and animal disease.

WNV was recognised as a human pathogen in Africa during the first half of the 20th century. Although several WNV fever epidemics were described, encephalitis as a consequence of human WN infection was rarely encountered prior to 1996; ~~but~~ since then, outbreaks of human West Nile encephalitis have been reported from Romania, Russia, Israel, North America, France, ~~and~~ Tunisia, Italy, and Greece ~~(Bin *et al*., 2001; Del Giudice *et al*., 2004; Hayes, 2001; Hubalek & Halouzka, 1999; Zeller & Schuffenecker, 2004)~~. During the 1960s, West Nile viral encephalitis of horses was reported from Egypt and France (Panthier *et al*., 1966; Schmidt & El Mansoury, 1963). Since 1998, outbreaks of equine WNV encephalitis have been reported from Argentina, Canada, France, Israel, Italy, ~~Canada,~~ Morocco, Spain, and the United States of America~~, Israel and Morocco (Cantile *et al*., 2000; Hayes *et al*., 2005; Murgue *et al*., 2001; Ostlund *et al*., 2000)~~. In ~~the Western Hemisphere,~~ 2011, ~~the largest~~ an outbreak of equine encephalitis due to Kunjin virus, a Lineage 1 WNV, was reported in Australia (Frost *et al,* 2012). There was no evidence of disease in humans or birds caused by this virus.

The occurrence of disease in humans and animals along with bird and mosquito surveillance for WNV activity demonstrate that the virus range has dramatically expanded ~~from a discrete region along the East Coast of New York State to include the contiguous States of the United States of America (USA), Canada, Mexico, the Caribbean islands, Central America, Argentina, Columbia and Venezuela~~ including North, Central and South America as well as Europe and countries facing the Mediterranean Basin ~~(Davis *et al*., 2005; Morales *et al*., 2006; Ostlund *et al*., 2000; USDA, 2010). Other than in the United States and Canada, the introduction of West Nile virus in the Western Hemisphere has not been characterised by large disease outbreaks or significant mortality in any species, possibly because of exposure to indigenous flaviviruses circulating in these regions~~.

The incubation period for equine WN encephalitis following mosquito transmission is estimated to be 3–15 days. A fleeting viraemia of low virus titre precedes clinical onset (Bunning *et al*., 2002; Schmidt & El Mansoury, 1963). WN viral encephalitis occurs in only a small per cent of infected horses; the majority of infected horses do not display clinical signs (Ostlund *et al*., 2000). The disease in horses is frequently characterised by mild to severe ataxia. Additionally, horses may exhibit weakness, muscle fasciculation and cranial nerve deficits (Cantile *et al*., 2000; Ostlund *et al*., 2000; 2001; Snook *et al*., 2001). Fever is an inconsistently recognised feature. Treatment is supportive and signs may resolve or progress to terminal recumbency. The mortality rate is approximately one in three clinically affected unvaccinated horses. Differential diagnoses in horses include other arboviral encephalidites (e.g. eastern, western or Venezuelan equine encephalomyelitis, Japanese encephalitis), equine protozoal myelitis (*Sarcocystis neurona*), equine herpesvirus-1, Borna disease and rabies.

Most species of birds can become infected with WNV; the clinical outcome of infection is variable. ~~Chickens and turkeys, are~~ Some species appear resistant ~~to disease, Outbreaks of~~ while others suffer fatal neurologic disease. Neurologic disease and death have been ~~reported in zoo birds in the USA and in~~ documented in domestic geese in Israel and Canada, and in many native and exotic zoo birds in the USA during the emergence of WNV (Austin *et al*., 2004; Steele *et al*., 2000). In Europe fatal neurological disease has been reported in wild birds (Zeller & Schuffenecker, 2004). WNV has been associated with sporadic disease in small numbers of other species, including squirrels, chipmunks, bats, dogs, cats, white-tailed deer, reindeer, sheep, alpacas, alligators and ~~a~~ harbour seals during intense periods of local viral activity.

There has been confirmed transmission of WNV in humans by blood transfusion, organ transfer and breast milk, but most human infections occur by natural transmission from mosquitoes. ~~but~~ Laboratory acquired infections have also been reported (Campbell *et al.,* 2002). ~~In clinically suspicious cases,~~ Laboratory manipulations ~~diagnostic specimens from all animals, particularly birds,~~ should be ~~handled~~ carried out at an appropriate biosafety and containment level (BSL) determined by biorisk analysis ~~3 following appropriate laboratory procedures~~ (see Chapter 1.1.3 *Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities*). (~~Richmond & McKinney, 1999 As a guide, samples taken from horses and other mammals with suspected WNV infection should be handled under BSL-2 conditions. Dissection of field collected birds is recommended at BSL-3 due to the potentially high levels of virus found in such samples. BSL-2 is suitable for processing field collected mosquito pools whereas BSL-3 containment, equipment, and facilities are recommended for all manipulations of WNV cultures and for experimental animal and vector studies.~~ No vaccines are available for use in humans.

~~United States Department of Health and Human Services, 2009). Containment level 2 is appropriate for handling diagnostic specimens from horses. There has been confirmed transmission of WNV in humans by blood transfusion, organ transfer and breast milk.~~

Due to the occurrence of inapparent WNV infections, diagnostic criteria must include a combination of clinical assessment and laboratory tests.

B. DIAGNOSTIC TECHNIQUES

Table 1. *Test methods available for the diagnosis of West Nile fever and their purpose*

Method	Purpose				
	Population freedom from infection	Individual animal freedom from infection	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Nested RT-PCR	–	++	++	–	–
Real time RT-PCR	=	++	++	=	=
Isolation in tissue culture	=	++	++	=	=
IgM capture ELISA	=	=	++	=	=
Plaque reduction neutralisation	++	=	±	++	++
Serum neutralisation	++	=	±	++	++
Immunohisto-chemistry	-	-	±	-	

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose.
Although not all of the tests listed as category +++ or ++ have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.
RT-PCR = reverse-transcriptase polymerase chain reaction; IgM = immunoglobin;
ELISA = enzyme-linked immunosorbent assay.

1. Identification of the agent

a) *In-vitro* and *in-vivo* culture

Attempts to detect virus from live, clinically ill horses are not usually successful due to the fleeting viraemia. Specimens for virus isolation include brain (particularly hindbrain and medulla) and spinal cord from deceased encephalitic horses (Ostlund *et al*., 2000; 2001); a variety of bird tissues including brain, heart or liver may be used with success (Steele *et al*., 2000). WNV ~~has~~ can also be~~en~~ isolated from ~~kidney but the tissue may be toxic to cell culture~~ mosquitoes. In general, virus isolates are obtained more easily from avian specimens and to a lesser extent from mosquitoes and horses.

Virus may be propagated in susceptible cell cultures, such as rabbit kidney (RK-13), African green monkey kidney (Vero), or pig kidney cells. Primary isolation in ~~or~~ embryonated chicken eggs or *Aedes albopictus* (C6/36) cell lines followed by passages in mammalian cells can also be used. ~~Intracerebral inoculations of newborn mice are less likely to yield virus isolates from mammalian tissues than cell culture methods.~~ More than one cell culture passage may be required to observe cytopathic effect (CPE). Confirmation of WNV isolates is achieved by indirect fluorescent antibody staining of infected cultures or nucleic acid detection methods (see below).

~~b) Immunological methods~~

~~Immunohistochemical (IHC) staining of formalin-fixed avian tissues is a reliable method for identification of WNV infection in birds. Brain, heart, kidney, spleen, liver, intestine, and lung are often IHC-positive tissues in infected birds. The success rate of IHC detection in positive birds is enhanced by the examination of multiple tissues. The specificity of identification (e.g. flavivirus specific or WNV specific) depends on the selection of detector antibody. The brain and spinal cord tissues of horses with WN viral encephalitis are inconsistently~~

~~positive in IHC tests; approximately 50% of equine encephalitis cases yield false-negative results. Failure to identify WNV antigen in equine central nervous system does not rule out infection.~~

b) Molecular methods – detection of nucleic acid ~~**Nucleic acid recognition methods**~~

~~Nucleic acid detection by reverse-transcription polymerase chain reaction (RT-PCR) significantly enhances~~ Several PCR methods have been described for the identification of WNV and some are available as commercial kits.~~ infected tissues, particularly when a nested PCR approach is applied to fresh, unfixed, equine brain and spinal cord specimens~~ Included here is a real-time RT-PCR with the capacity to detect both lineage 1 and lineage 2 WNV (Eiden *et al.,* 2010). Additionally, a conventional, gel-based RT-PCR designed to detect lineage 1 North American strains is described (Johnson *et al*., 2001). Both assays have been successfully employed with field collected samples. ~~The RT-nested PCR method to detect WNV nucleic acid encoding a portion of the E gene is described below. This method was developed using a 1999 North American isolate and has been successful in detecting WNV RNA in animal tissues during recent North American outbreaks. St Louis encephalitis virus is not detected by this method.~~ Lineage 1 WNV~~s~~ from ~~China (People's Rep. of),~~ France, Egypt, Israel, Italy, Kenya, Mexico and Russia demonstrate a highly conserved nucleotide sequence in the target region, regardless of species of origin (Lanciotti *et al*., 2000). ~~Analysis of sequence information for the Uganda 1937 Lineage 2 strain (GenBank M12294) in the region targeted by the PCR primers indicate that amplification of lineage 2 strains of WNV would not be expected. Other viruses from, the Japanese encephalitis serogroup have not been examined. Non-nested methods, including real-time PCR, pose less risk of laboratory cross-contamination and may be applied successfully to avian tissue samples (Lanciotti *et al*., 2000). A real-time RT-PCR has been described for the detection of WNV nucleic acid (Tewari *et al*., 2004). In order to standardise WNV molecular techniques a proficiency study based on formalin-fixed tissues was developed, administered and reported (Niedrig *et al*., 2006). Tissues selected for PCR are the same as those selected for virus isolation attempts.~~ While the laboratory practices required to avoid contamination in a nested method are stringent, there is higher sensitivity for detection of North American strains of WN RNA with the conventional nested procedure, particularly in equine field samples. The efficiency of the conventional RT-PCR to detect other WNV lineages is not known. In view of the continued evolution and possible emergence of new WNV strains, it is important that the designs of PCR tests are constantly monitored and updated when necessary. Samples appropriate for WNV RT-PCR include mammalian brain, avian brain, kidney, heart, liver, intestine, and insect pools. In any PCR assay it is imperative to include positive and no-template controls. For the nested RT-PCR measures must be employed to avoid contamination with amplified material during the transfer of the outer primer product to the nested reaction tubes. For any PCR reaction to be valid, the control reactions must fall within the expected range.

~~• **Reverse-transcription nested polymerase chain reaction (RT-nPCR) procedure**~~

~~The RT-nPCR described here includes several procedures: extraction of RNA, reverse transcription to generate DNA from RNA and first stage PCR, second stage PCR using 'nested' primers and, finally, detection of the appropriately sized amplicon by gel electrophoresis. WNV E protein gene regions of 445 bp (base pairs) and 248 bp are amplified in the first stage and nested procedures, respectively. The kits and reagents described below are provided as examples. Equivalent products may be available from other sources. Extreme care in handling all materials and inclusion of proper controls are essential to ensure accurate results. The precautions to be taken have been covered in Chapter 1.1.5 *Principles and methods of validation of diagnostic assays for infectious diseases*. Duplicate samples of each diagnostic specimen should be processed and tested to increase confidence in test results. Use appropriate precautions when handling hazardous reagents such as ethidium bromide.~~

• **Extraction of viral RNA**

~~From 50 to 100 mg of tissue, extract total RNA using Trizol® reagent (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. Also extract total RNA from WNV control stock virus containing 10–100 tissue culture infective dose ($TCID_{50}$) per 100 µl volume.~~ Several commercial kits are available for RNA extraction. Select a kit appropriate for the type of sample and follow the manufacturer's recommendations.

~~• **Reverse transcription and first stage PCR**~~

~~First stage primers:~~
~~1401: 5'-ACC-AAC-TAC-TGT-GGA-GTC-3'~~
~~1845: 5'-TTC-CAT-CTT-CAC-TCT-ACA-CT-3'~~

• **Real-time reverse transcription PCR**

The following method was developed by Eiden *et al.* (2010) for concurrent identification of lineage 1 and lineage 2 WNV. Strain identification may be achieved by sequencing of the resultant amplicon and alignment with WNV reference strains. The procedure has been slightly modified from the published

method and included here are the primers and probe directed to the NS2A region of the WNV genome. The assay may be performed with a commercial kit of choice that provides the expected amplification of the controls. The cycling parameters must be adjusted to conform to the kit requirements. Primer and probe concentrations may be adjusted to achieve optimal results. An internal control and appropriate control primers and probe may be included to confirm valid test conditions.

Primers/probe (NS2A region of genome):

Forward primer: GGG-CCT-TCT-GGT-CGT-GTT-C
Reverse primer: GAT-CTT-GGC-YGT-CCA-CCT-C
Probe: FAM-CCA-CCC-AGG-AGG-TCC-TTC-GCA-A-BHQ

Per sample, prepare 20 µl volume of the RT-PCR reagents (per kit instructions) containing a 0.9 µM concentration of each primer and a 0.25 µM probe concentration. Dispense 20 µl of the mixture into each sample PCR tube or well. Add 5.0 µl of the extracted RNA sample, seal the tube/plate, and place in the thermocycler. Run the samples under the conditions described for the kit in use, beginning with a reverse transcription incubation, followed by 45 cycles of amplification. Ct values of 37 or less are considered positive for WNV. Ct values of 37.1 through 42 are considered suspect, and should be repeated. Values higher than 42 are negative. For the PCR to be valid, the Ct values of the positive controls should fall within the expected range. No-template controls must be negative.

- **Conventional reverse transcription PCR**

The following method was developed for detection of lineage 1 WNV (Johnson *et al.,* 2001). The procedure may be conducted as a one-step RT-PCR using the outer primers only, or as a nested assay. The nested assay is the most sensitive RT-PCR and is recommended for testing of mammalian brain tissues or other samples that may contain a low amount of virus. The target of this RT-PCR is the E region of the WNV genome. The assay may be performed with a commercial kit of choice that provides the expected amplification of the controls. The cycling parameters must be adjusted to conform to the kit requirements.

Outer primers:

1401F: ACC-AAC-TAC-TGT-GGA-GTC
1845R: TTC-CAT-CTT-CAC-TCT-ACA-CT

Nested primers:

1485F: GCC-TTC-ATA-CAC-ACT-AAA-G
1732R: CCA-ATG-CTA-TCA-CAG-ACT

Per sample, prepare 45 µl volume of the RT-PCR reagents (per kit instructions) containing a 0.6 µM concentration of each outer primer. Dispense 45 µl of the mixture into each sample PCR tube. Add 5.0 µl of the extracted RNA sample and place the tubes in the thermocycler. Run the samples under the conditions described for the kit in use, beginning with a reverse-transcription incubation, followed by 35 cycles of amplification. For the nested reaction, prepare a similar reaction mixture (without reverse transcriptase) of 49 µl per sample containing the nested primers and dispense into PCR tubes. Transfer 1.0 µl of the outer primer amplification product to the nested tube and place in the thermocycler. Use caution when transferring the amplification product to avoid cross contamination of samples. Perform 35 amplification cycles per kit recommendations. Following amplification, mix 8–10 µl of each PCR product with a loading buffer containing a DNA stain. Load the mixture into a gel and analyse by agar gel electrophoresis and ultraviolet visualization. WNV-positive samples will be identified by a 445 base-pair band (outer primer amplification only) and/or a 248 base pair band (nested amplification). For the PCR reaction to be valid, positive controls must have the bands of the expected size, and no template controls should have not have bands. PCR amplicons may be sequenced for identity confirmation.

~~i) Suspend extracted RNA samples in 12 µl RNase-free water.~~

~~ii) Incubate at 70°C for 10 minutes.~~

~~iii) Add 2 µl of each RNA sample to 48 µl of RT-PCR mixture containing a final composition of:~~
~~10 mM Tris/HCl, pH 8.3~~
~~50 mM KCl~~
~~2.0 mM $MgCl_2$~~
~~0.8 mM deoxynucleoside triphosphate (dNTP) pool~~
~~25 units M-MLV (Moloney murine leukaemia virus) RT~~
~~1.25 units RNase inhibitor~~
~~1.25 units AmpliTaq Gold™ (Applied Biosystems, Foster City, CA, USA)~~
~~37.5 pmol of the first stage primers.~~

~~Include 'no RNA' controls using 2 µl RNase-free water in place of denatured RNA.~~

~~iv) Incubate reaction tubes at 45°C for 45 minutes.~~

~~v) Incubate reaction tubes at 95°C for 11 minutes.~~

~~vi) PCR amplification through 35 cycles:~~
~~Denaturation at 95°C for 30 seconds,~~
~~Primer annealing at 55°C for 45 seconds,~~
~~Primer extension at 72°C for 60 seconds (for the 35th cycle, primer extension at 72°C for 5 minutes).~~

~~vii) Hold samples at 4°C until second stage PCR.~~

~~• **Second stage (nested) PCR**~~

~~Second stage primers:~~
~~1485: 5' GCC TTC ATA CAC ACT AAA G 3'~~
~~1732: 5' CCA ATG CTA TCA CAG ACT 3'~~

~~i) For each sample and control, add 1.5 µl of the first-stage amplification product to 48.5 µl of PCR mixture with a final composition of:~~
~~10 mM Tris/HCl, pH 8.3~~
~~50 mM KCl~~
~~2.0 mM $MgCl_2$~~
~~0.8 mM deoxynucleoside triphosphate (dNTP) pool~~
~~1.25 units AmpliTaq Gold™ (Applied Biosystems, Foster City, CA, USA)~~
~~37.5 pmol each of the nested primers.~~

~~ii) Incubate reactions tubes at 95°C for 11 minutes.~~

~~iii) PCR amplification through 35 cycles:~~
~~Denaturation at 95°C for 30 seconds,~~
~~Primer annealing at 55°C for 45 seconds,~~
~~Primer extension at 72°C for 60 seconds (for the 35th cycle, primer extension at 72°C for 5 minutes).~~

~~iv) Hold samples at 4°C or –20°C until electrophoresis.~~

~~• **Analysis of PCR products by gel electrophoresis**~~

~~i) Prepare a 2.5% NuSieve® 3/1 (FMC Bioproducts, Rockland, Maine, USA) agarose solution in 0.045 mM Tris/borate, pH 8.6, 1.5 mM EDTA (ethylene diamine tetra-acetic acid) (1 × TBE buffer). Boil the agarose on a hotplate or in a microwave oven until completely dissolved. Cool the agarose to 45–55°C. Add 5.0 µl ethidium bromide solution (10 mg/ml) per 100 ml warm agarose and pour agarose gel with comb. Allow to solidify then remove comb.~~

~~ii) Add 30 µl ethidium bromide solution (10 mg/ml) per 600 ml 1 × TBE tank buffer. Position gel in apparatus and fill buffer tanks.~~

~~iii) Mix 15 µl of each sample and control with 5 µl gel loading solution (e.g. Sigma product G-2526, St Louis, MO, USA). Include 100 bp DNA ladder (e.g. Life Technologies, Grand Island, NY, USA product 15268-019, range 100–1500 bp) in at least one gel well. Load samples into agar wells and electrophorese at 65–75 volts until the gel loading dye has travelled approximately two-thirds the length of the gel.~~

~~iv) Visualise and photograph gel under ultraviolet illumination.~~

~~• **Interpretation of the test**~~

~~For the PCR test to be valid, the positive controls must show the appropriate size band (248 bp). The 'no RNA' controls should have no bands. Samples are considered to be positive if there is a band of the same size as the positive control. Duplicate samples should both show the same reaction. If there is a disparity, the test should be repeated, starting with extraction from tissue. If further validation is required, the final nested PCR product can be sequenced and compared with the published sequences of WNV from GenBank.~~

b) Antigen detection – immunolabelling techniques ~~Immunological methods~~

Immunohistochemical (IHC) staining of formalin-fixed avian tissues is a reliable method for identification of WNV infection in birds. Brain, heart, kidney, spleen, liver, intestine, and lung are often IHC-positive tissues in infected birds. The success rate of IHC detection in positive birds is enhanced by the examination of multiple tissues. The specificity of identification (e.g. flavivirus specific or WNV specific) depends on the selection of detector antibody. The brain and spinal cord tissues of horses with WN viral encephalitis are inconsistently positive in IHC tests; ~~approximately 50% of~~ many equine encephalitis cases yield false-negative results. Failure to identify WNV antigen in equine central nervous system does not rule out infection. For further advice, consult OIE Reference Laboratories.

2. Serological tests

Antibody can be identified in equine serum by IgM capture enzyme-linked immunosorbent assay (IgM capture ELISA), haemagglutination inhibition (HI), IgG ELISA, ~~or~~ plaque reduction neutralisation (PRN), and microtitre virus neutralisation (VN) (Beaty *et al*., 1989; Hayes, 1989). The IgM capture ELISA described below is particularly useful to detect equine antibodies resulting from recent natural exposure to WNV. Equine WNV-specific IgM antibodies are usually detectable from 7–10 days post-infection to 1–2 months post-infection. Most horses with WN encephalitis test positive in the IgM capture ELISA at the time that clinical signs are first observed. WNV neutralising antibodies are detectable in equine serum by 2 weeks post-infection and can persist for more than 1 year. The ELISA, HI, VN and PRN methods are most commonly used for identifying WNV antibody in avian serum. In some serological assays, antibody cross-reactions with related flaviviruses, such as St Louis encephalitis virus or Japanese encephalitis virus, will be encountered. The PRN test is the most specific among WNV serological tests; when needed, serum antibody titres against related flaviviruses can be tested in parallel. Finally, WN vaccination history must be considered in interpretation of serology results, particularly in the PRN and VN tests and IgG ELISA. An IgM capture ELISA may be used to test avian or other species provided that species-specific capture antibody is available (e.g. anti-chicken IgM). The PRN test is applicable to any species, including birds.

a) ELISA and related methods

- **Equine IgM capture ELISA**

WNV and negative control antigens for the IgM capture ELISA may be prepared from mouse brain (see chapter 2.5.5), tissue culture or recombinant cell lines (Davis *et al*., 2001). Commercial sources of WNV testing reagents are available in North America. Characterised equine control serum, although not an international standard, can be obtained from the National Veterinary Services Laboratories, Ames, Iowa, USA. Virus and negative control antigens should be prepared in parallel for use in the ELISA. Antigen preparations must be titrated with control sera to optimise sensitivity and specificity of the assay. Equine serum samples are tested at a dilution of 1/400 and equine cerebrospinal fluid samples are tested at a dilution of 1/2 in the assay. To ensure specificity, each serum sample is tested for reactivity with both virus antigen and control antigen.

- **Test procedure**

i) Coat flat-bottom 96-well ELISA plates ~~(e.g. Immulon 2HB, Dynex Technologies, Chantilly, VA, USA)~~ with 100 µl/well anti-equine IgM diluted in 0.5 M carbonate buffer, pH 9.6, according to the manufacturer's suggested dilution for use as a capture antibody.

ii) Incubate plates overnight at 4°C in a humid chamber. Coated plates may be stored for several weeks in a similar manner.

iii) Prior to use, wash plates twice with 200–300 µl/well 0.01 M phosphate buffered saline, pH 7.2, containing 0.05% Tween 20 (PBST).

iv) Block plates by adding 300 µl/well freshly prepared 5% nonfat dry milk in PBST and incubate 60 minutes at room temperature. After incubation, remove blocking solution and wash plates three times with PBST.

v) Test and control sera are diluted 1/400 (cerebrospinal fluid is diluted 1/2) in PBST and 50 µl/well of each sample is added to duplicate sets of wells (total of four wells per sample) on the plate. Include control positive and negative sera prepared in the same manner as samples.

vi) Cover the plates and incubate 75 minutes at 37°C in a humid chamber.

vii) Remove serum and wash plates three times in PBST.

viii) Dilute virus and negative control antigens in PBST and add 50 µl of virus antigen to one set of wells per test and control sera and add 50 µl normal antigen to the second set of wells per test and control sera.

ix) Cover the plates and incubate overnight at 4°C in a humid chamber.

x) Remove antigens from the wells and wash the plates three times in PBST.

xi) Dilute horseradish peroxidase conjugated anti-*Flavivirus* monoclonal antibody[1] in PBST according to manufacturer's directions and add 50 µl per well.

xii) Cover the plates and incubate at 37°C for 60 minutes.

xiii) Remove conjugate and wash plates six times in PBST.

1 Available from the Centers for Disease Control and Prevention, Biological Reference Reagents, 1600 Clifton Road NE, Mail Stop C21, Atlanta, Georgia, 30333, USA.

xiv) Add 50 µl/well freshly prepared ABTS (2,2'-azino-di-[3-ethyl-benzthiazoline]-6-sulphonic acid) substrate with hydrogen peroxide (0.1%) and incubate at room temperature for 30 minutes.

xv) Measure absorbance at 405 nm. A test sample is considered to be positive if the absorbance of the test sample in wells containing virus antigen is at least twice the absorbance of negative control serum in wells containing virus antigen and at least twice the absorbance of the sample tested in parallel in wells containing control antigen.

- **Indirect and competitive ELISAs**

Numerous indirect and competitive commercial and in-house ELISAs have been developed and are used to detect WNV antibodies. While competitive assays are applicable for sera of all species, indirect assays require enzyme-labelled species specific antibodies. Both ELISA techniques lack specificity as they cross react with antibodies directed to other flaviviruses especially those of the Japanese encephalitis serogroup. They are very useful for epidemiological and surveillance purposes as well as a screening method. A positive ELISA result however should be confirmed by a more specific test such as VN or PRNT. Most indirect or competitive ELISAs detect antibodies of any immunoglobulin class (IgM, IgG, etc.). ~~In contrast to the IgM capture ELISA,~~ Vaccinated horses often test positive in the indirect or competitive ELISs.

b) Neutralisation

- **Plaque reduction neutralisation (applicable to serum from any species)**

The PRN test is performed in Vero cell cultures in either 25 cm^2 flasks or 6-well plates. The sera can be screened at a 1/10 and 1/100 final dilution or may be titrated to establish an endpoint. A description of the test as performed in 25 cm^2 flasks using 100 plaque-forming units (PFU) of virus is as follows.

Prior to testing, serum is heat inactivated at 56°C for 30 minutes and diluted (e.g. 1/5 and 1/50) in media. Virus (200 pfu per 0.1 ml) working dilution is prepared in media containing 10% guinea-pig complement. Equal volumes of virus and serum are mixed and incubated at 37°C for 75 minutes before inoculation of 0.1 ml on to confluent cell culture monolayers. The inoculum is adsorbed for 1 hour at 37°C, followed by the addition of 4.0 ml of primary overlay medium. The primary overlay medium consists of two solutions that are prepared separately. Solution I contains 2 × Earle's Basic Salts Solution without phenol red, 4% fetal bovine serum, 100 µg/ml gentamicin and 0.45% sodium bicarbonate. Solution II consists of 2% Noble agar that is sterilised and maintained at 47°C. Equal volumes of solutions I and II are adjusted to 47°C and mixed together just before use. The test is incubated for 72 hours at 37°C. A second 4.0 ml overlay prepared as above, but also containing 0.003% neutral red is applied to each flask. Following a further overnight incubation at 37°C, the number of virus plaques per flask is assessed. Endpoint titres are based on 90% reduction compared with the virus control flasks, which should have about 100 plaques.

- **Virus neutralisation – microtitre format**

The microtitre VN assay is capable of identifying and quantifying antibodies against WNV present in test samples. Its performance is comparable to the PRNT; however, it requires less sample volume than PRNT and is more suitable when only small volumes of samples are available (Weingartl *et al.*, 2003). Appropriate precautions are necessary to prevent human exposure when using live WNV in unsealed microtitre plates.

- **Test procedure**

The cell line commonly used is African green monkey kidney (Vero). The VN test requires 3–5 days to be completed.

i) Twenty-five µl of several sera dilutions, starting from 1/5 to 1/640, are added to each test well of a sterile flat-bottomed microtitre plates and each mixed with an equal volume of 100 $TCID_{50}$ of WNV reference virus. They are incubated at 37°C in 5% CO_2.

ii) After 1 hour incubation, approximately 10^4 VERO cells are added per well in a volume of 50 µl, of Minimal Essential Medium containing antibiotics and, after incubation for 3–5 days, the test is read using an inverted microscope

iii) Wells are scored for the degree of CPE observed. A sample is considered positive when it shows more than 90% of CPE neutralisation at the lowest dilution (1:10). The serum titre represents the highest serum dilution capable to neutralise more than 90% CPE in the tissue culture.

A VN titre greater or equal 1/10 is usually considered specific for WNV. However it should be noted that birds and mammals may show cross reactions at this level after infection with, or vaccination against, Japanese encephalitis or St Louis encephalitis viruses.

~~Standard microneutralisation or microtitre plaque reduction neutralisation assays may be more suitable when small volumes of samples are available (Weingartl *et al.*, 2003).~~

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

1. Background

In ~~February~~ 2003, the United States Department of Agriculture (USDA) issued a license for a formalin-inactivated WNV vaccine derived from tissue culture for use in horses. ~~This was followed in December 2003, by a USDA licensed~~ The European Committee for Medicinal Products for Veterinary Use (CVMP) approved this product in 2008. In 2011, the product was conditionally licensed by USDA for use in alligators. ~~In 2003, the USDA licensed a live canarypoxvirus vectored WNV vaccine for use in horses.~~ In 2004, an inactivated human cell line-derived WNV vaccine ~~developed by Crucell NV (the Netherlands) and Kimron Veterinary Institute (Israel)~~ obtained a market authorisation in Israel as a veterinary vaccine for geese. ~~In July 2005, the USDA issued the first fully licensed WNV DNA vaccine for animals in the USA. The vaccine contains genes for two WNV proteins, and therefore, does not contain any whole WNV, live or killed. In late 2006, a chimeric vaccine, based on a yellow fever virus vector, was licensed by USDA for use in horses. The CVMP approved a recombinant canarypox virus WNV product in 2011.~~ Information on Biotechnology-derived vaccines have also been licensed, as detailed in section C.3 below. These vaccines have demonstrated sufficient efficacy and safety in adequately vaccinated horses. Vaccination may be helpful in preventing neurological signs associated with WNV infection.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements.

~~1. Seed management~~

2. Outline of production and minimum requirements for vaccines

a) Characteristics of the seed

See chapter 1.1.6 for general requirements for master seeds and allowable passages for vaccine production.

i) *Biological characteristics of the master seed* (*MS*)

The isolate of WNV used as the master seed virus (MSV) for vaccine production must be accompanied by documentation describing its origin and passage history. ~~The isolate must be safe in host animals at the intended age of vaccination and provide protection after challenge.~~

Inactivated vaccines: Virulent virus potentially may be used in inactivated vaccines, provided that manufacturing methods ensure complete inactivation of the MSV. The completed vaccine must be safe in host animals at the intended age of vaccination and provide protection after challenge.

Live vaccines: The MSV must be safe in host animals at the most susceptible age for infection. It should not increase in virulence or undergo detectable genetic changes with repeated passage through host animals. Ideally, the MSV should not be shed from vaccinated animals into the environment; otherwise, shedding should be minimal and transient. The MSV should not adversely impact non-target species with which a vaccinated animal may have contact. The completed vaccine must provide protection after challenge.

Recombinant vaccines: Recombinant vaccines may be live or inactivated, and recombinant MSVs are subject to the same guidelines as conventional MSVs. In addition, recombinant MSVs expressing foreign genes should stably produce the foreign antigens.

DNA vaccines: The MS is the host organism (e.g. *Escherichia coli*) that expresses the plasmid used in the vaccine. The completed DNA vaccine is subject to the same requirements as listed above.

ii) *Quality criteria* (*sterility, purity, freedom from extraneous agents*)

The MSV must be tested for purity, identity, and freedom from extraneous agents at the time before it is used in the manufacture of vaccine. The MSV must be free from bacteria, fungi and mycoplasma. It also must be free of extraneous viruses, including equine herpesvirus, equine adenovirus, equine viral arteritis virus, bovine viral diarrhoea virus, reovirus, and rabies virus by the fluorescent antibody technique. The MSV must be free from extraneous virus by CPE and haemadsorption on the Vero cell line and an embryonic equine cell type.

iii) *Validation as a vaccine strain*

In an immunogenicity trial, the MSV at the highest passage level intended for production must protect susceptible horses against a virulent challenge isolate found in the geographical area of intended vaccine use. Ideally the challenge isolate and MSV should be heterologous. MSVs intended for use in live vaccines also must be tested for innocuity in susceptible non-target species, such as birds.

iv) *Emergency procedure for provisional acceptance of new master seed virus (MSV) in the case of an epizootic*

In the event that an emerging strain or variant of WNV results in an emergency epizootic situation that cannot be controlled by currently available vaccines, provisional acceptance of a new MSV should be considered, ideally for use in an inactivated vaccine. Such acceptance should be based on a risk analysis of potential contamination of the new MSV with extraneous agents. This risk assessment should consider the source and passage history of the MSV and characteristics of the vaccine manufacturing process, including the nature and concentration of the virus inactivant.

b) Method of manufacture ~~culture~~

i) *Procedure*

The MSV should be propagated in cell lines known to support the growth of WNV. See chapter 1.1.6 for additional guidance on the preparation and testing of master cell stocks. Cell lines should be free from extraneous viruses, bacteria, fungi, and mycoplasma. Viral propagation should not exceed five passages from the MSV, unless further passages prove to provide protection in the host animal.

The susceptible cell line is seeded into suitable vessels. Minimal essential medium, supplemented with fetal bovine serum (FBS), may be used as the medium for production. Incubation is at 37°C.

Cell cultures are inoculated directly with WNV working virus stock, which is generally 1 to 4 passages from the MSV. Inoculated cultures are incubated for 1–8 days before harvesting the culture medium. During incubation, the cultures are observed daily for CPE and bacterial contamination.

Inactivated vaccines may be chemically inactivated with either formalin or binary ethylenimine and mixed with a suitable adjuvant. The duration of the inactivation period is based on demonstrated inactivation kinetics.

DNA vaccine expression cassettes are amplified in *E. coli* (or other suitable vector). Purified plasmids are formulated into a vaccine.

ii) *Requirements for ingredients*

All ingredients used in the manufacture of WNV vaccine should be defined in approved manufacturing protocols and consistent from batch to batch. See chapter 1.1.6 for general guidance on ingredients of animal origin. Ingredients of animal origin should be sourced from a country with negligible risk for transmissible spongiform encephalopathies (TSEs).

iii) *In-process controls*

Production lots of WNV must be titrated in tissue culture before inactivation to standardise the product. Low-titred lots may be concentrated or blended with higher-titred lots to achieve the correct titre.

Inactivated WNV lots must be tested for completeness of inactivation. Ideal protocols incorporate concentration and amplification steps, to enhance detection of residual live virus.

Production lots of DNA are quantified by analytical methods and characterised before standardisation and blending at the correct DNA content. Production lots must not exceed the highest acceptable level of lipopolysaccharide (LPS) content, based on safety testing.

iv) *Final product batch tests*

Sterility

Inactivated and live vaccine samples are examined for bacterial and fungal contamination. The volume of medium used in these tests should be enough to nullify any bacteriostatic or fungistatic effects of the preservatives in the product. To test for bacteria, ten vessels, each containing a minimum of 120 ml of soybean casein digest medium, are inoculated with 0.2 ml from ten final-container samples. The ten vessels are incubated at 30–35 from 10 days and observed for bacterial growth. To test for fungi, ten vessels, each containing a minimum of 40 ml of soybean casein digest medium, are inoculated with 0.2 ml from ten final-container samples. The vessels are incubated at 20–25 from ten days and observed for fungal growth. Individual countries may have other requirements.

Identity

Separate batch tests for identity should be conducted if the batch potency test, such as tissue-culture titrations of live virus vaccines, does not sufficiently verify the identity of the agent in the vaccine. Identity tests may include fluorescent antibody or serum neutralisation assays.

Additionally, if all-in, all-out manufacturing processes and sanitation are not used and more than one agent is propagated in a laboratory, identity tests shall demonstrate that no other vaccine strain is present.

Safety

Batch safety tests may be conducted in guinea pigs, mice, or host animals. The product should be administered according to label recommendations in host animals. Individual countries may require dose overage (e.g. 2×–10×). No systemic or local adverse reactions should occur after vaccination.

The requirement for an *in-vivo* batch safety test may be exempted if the safety of the product is demonstrated and approved in the registration dossier and production is consistent with that described in chapters 1.1.6 and 1.1.8.

Batch potency

Killed virus vaccines may use host animal or laboratory animal vaccination/serology tests or vaccination/challenge tests to determine potency of the final product. In vitro techniques to compare a standard with the final product are also acceptable in determining the relative potency of a product (USDA, 2011). The standard should be shown to be protective in the host animal.

Live viral products are titred in cell cultures to determine the potency of the final product. Compared to the minimum protective dose established in the immunogenicity trial, the batch release potency titre should include overages for batch test variability and loss of potency over product dating. In the absence of specific data to support an alternative, overages of 0.7 $\log_{10}$ and 0.5 $\log_{10}$, respectively, are recommended.

DNA vaccines may be tested for bioactivity and DNA content using parallel-line direct quantification methods that compare a standard preparation to the final product.

c) Requirements for authorisation/registration/licensing

i) *Manufacturing process*

For registration of WNV vaccine, all relevant details concerning manufacture of the vaccine, in-process controls, and quality control testing (see Section C.2.a and b) should be submitted to the authorities. Test results shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume

ii) *Safety requirements*

The inherent safety of the vaccine strain, if it will be used in the manufacture of a live vaccine, is tested at the Master Seed level:

Target and non-target animal safety: The Seed should be tested in host animals of the most susceptible age. The animals should be monitored for clinical disease and virus shed/spread. The organism dose should be no less than that expected in completed vaccine. Individual countries may have overdose requirements. If the vaccine will be intended for use in specific subpopulations (e.g., pregnant animals), they also should be included in target animal safety studies. Additionally, similar studies should be conducted in susceptible non-target species (e.g. birds).

Reversion-to-virulence for attenuated/live vaccines and environmental considerations: Demonstrate that repeated *in-vivo* passage of the MSV does not increase virulence. (For additional guidance see Section: *Increase in Virulence Tests* in chapter 1.1.6.)

The final vaccine formulation (inactivated or live) should be tested in a limited number of target animals prior to a larger-scale field study. The final vaccine formulation should not cause adverse reactions.

Field safety studies should be conducted before any vaccine receives final approval. Generally, two serials should be used, in three different geographical locations under typical animal husbandry conditions, and in a minimum of 600 animals. The vaccine should be administered according to label recommendations (including booster doses) and should contain the maximum permissible amount of WNV antigen. (If no maximum antigen content is specified, serials should be of anticipated typical post-marketing potency.) About one-third of the animals should be at the minimum age recommended for vaccination.

Precautions (*hazards*)

Vaccine should be identified as harmless or pathogenic for vaccinators. Manufacturers should provide adequate warnings that medical advice should be sought in the case of self-injection. Warnings should be included on the product label/leaflet so that the vaccinator is aware of any danger.

iii) *Efficacy requirements*

To register a WNV vaccine, a batch produced according to the standard method and containing the minimum amount of antigen or potency value must provide protection against virulent challenge in the minimum-aged animal recommended for vaccination. Each future commercial batch shall be tested before release to ensure it has at least the same potency demonstrated by the batch used for the efficacy study.

WNV vaccine efficacy is often estimated in vaccinated horses by evaluating post-challenge viraemia and/or neurological signs (e.g. muscle fasciculation, ataxia, seizures). Efficacy is estimated in alligators by evaluating post-challenge viraemia and/or lymphohistiocytic skin (pix) lesions. Twenty-five vaccinates and ten placebo-vaccinated controls are recommended.

Horses may be challenged intrathecally or via controlled exposure to infected mosquitoes. Alligators may be challenged subcutaneously. All animals should be monitored for 14–21 days after challenge. Viraemia should be evaluated qualitatively (i.e., detectable presence or absence) by validated assays. The vaccination effect may be assessed by calculating prevented fraction with a 95% confidence interval. Individual countries may have minimum efficacy requirements, but in no case should the lower confidence interval include zero.

iv) *Vaccines permitting a DIVA strategy* (*detection of infection in vaccinated animals*)

Vaccination is only recommended for horses and alligators in WN-positive areas. Vaccinated horses may develop a serological titre that may interfere with the ability to export the horse.

v) *Duration of immunity*

Studies to determine a minimum duration of immunity should be conducted before the vaccine receives final approval. Duration of immunity should be demonstrated in a manner similar to the original efficacy study, challenging animals at the end of the claimed period of protection. At a minimum, the duration should be for the length of the mosquito season in seasonally infected areas. It may be desirable to demonstrate longer immunity for animals at higher risk and in infected areas with year-round mosquito activity.

vi) *Stability*

Live and inactivated vaccines are typically assigned an initial dating of 18 or 24 months, respectively, before expiry. Real-time stability studies should be conducted to confirm the appropriateness of all expiration dating. Product labelling should specify proper storage conditions.

3. Vaccines based on biotechnology

In 2003, the USDA licensed a live canarypoxvirus vectored WNV vaccine for use in horses. In 2005, the USDA issued the first fully licensed WNV DNA vaccine for animals in the USA. The vaccine contains genes for two WNV proteins, and therefore, does not contain any whole WNV, live or killed. In late 2006, a chimeric vaccine, based on a yellow fever virus vector, was licensed by USDA for use in horses. The CVMP approved a recombinant canarypox virus WNV product in 2011. In addition to meeting requirements for efficacy, potency, purity and safety, recombinant seeds must undergo a risk analysis. The conclusion of the risk analysis must be that the vaccine does not have significant environmental impact.

~~The WNV should be propagated in cell lines known to support the growth of WNV. Cell lines should be free from extraneous viruses, bacteria, fungi, and mycoplasma. Viral propagation should not exceed five passages from the master seed virus (MSV), unless further passages prove to provide protection in the host animal.~~

~~c) Validation as a vaccine~~

~~The MSV should be free from bacteria, fungi and mycoplasma. The MSV must be tested for and be free of extraneous viruses, including equine herpesvirus, equine adenovirus, equine viral arteritis virus, bovine viral diarrhoea virus, reovirus, and rabies virus by the fluorescent antibody technique. The MSV must be free from extraneous virus by CPE and haemadsorption on the Vero cell line and an embryonic equine cell type.~~

~~In an immunogenicity trial, the MSV at the highest passage level intended for production must protect susceptible horses against a virulent challenge strain. A statistically significant number of vaccinated horses~~

must be protected from viraemia when compared with the controls. Field trial studies should be conducted to determine the safety of the vaccine.

2. Method of manufacture

The susceptible cell line is seeded into suitable vessels. Minimal essential medium, supplemented with fetal bovine serum (FBS), is used as the medium for production. Incubation is at 37°C.

Cell cultures are inoculated directly with WN working virus stock, which is generally from 1 to 4 passages from the MSV. Inoculated cultures are incubated for 1–8 days before harvesting the culture medium. During incubation, the cultures are observed daily for CPE and bacterial contamination.

Killed virus vaccines are chemically inactivated with either formalin or binary ethylenimine and mixed with a suitable adjuvant.

The DNA vaccine expression cassette is amplified in *Escherichia coli* using a plamid vector cutting out plasmid backbone and purified for formulation into a vaccine.

3. In-process control

Production lots of WNV must be titrated in tissue culture for standardisation of the product. Low-titred lots may be concentrated or blended with higher-titred lots to achieve the correct titre.

Production lots of DNA are quantified by analytical methods and characterised before standardisation and blending at the correct DNA content. The highest level of lipopolysaccharide (LPS) contamination of the DNA vaccine is 100 EU/dose (EU = endotoxin units).

4. Batch control

Final container samples are tested for purity, safety and potency.

a) Purity

Samples are examined for bacterial and fungal contamination. To test for bacteria, ten vessels, each containing 120 ml of soybean casein digest medium, are inoculated with 0.2 ml from ten final-container samples. The ten vessels are incubated at 30–35°C for 14 days and observed for bacterial growth. To test for fungi, ten vessels, each containing 40 ml of soybean casein digest medium, are inoculated with 0.2 ml from ten final-container samples. The vessels are incubated at 20–25°C for 14 days and observed for fungal growth.

b) Safety

Safety tests can be conducted in a combination of guinea pigs, mice or horses. Field safety studies should be conducted before the vaccine receives final approval. Generally, two serials should be used, in three different geographical locations, and a minimum of 600 animals. About one-third of the animals should be at the minimum age recommended for vaccination (correlated to efficacy). If the final product is a modified-live vaccine, additional safety testing of the MSV is required to demonstrate a lack of virulence.

c) Potency

Killed virus vaccines may use host animal or laboratory animal vaccination/serology tests or vaccination/challenge tests to determine potency of the final product. Parallel-line assays using ELISA antigen-quantifying techniques to compare a standard with the final product are acceptable in determining the relative potency of a product. The standard should be shown to be protective in the host animal (USDA, 1998). Live viral products are titred in cell cultures to determine the potency of the final product. The final release potency titre should include an additional 0.7 $\log_{10}$ for test variability and 0.5 $\log_{10}$ for end-of-dating stability than the minimum protective dose established in the immunogenicity trial.

DNA vaccines are tested for bioactivity and DNA content using parallel-line direct quantification methods that compare a standard preparation to the final product.

d) Duration of immunity

~~Duration of immunity studies are conducted before the vaccine receives final approval. The duration should be for the length of the mosquito season in the infected areas. For animals at higher risk and in infected areas with year-round mosquito activity, more frequent vaccine boosters may be advised.~~

~~e) Stability~~

~~All vaccines are initially given 24 months before expiry. Real-time stability studies are conducted to confirm the appropriateness of all expiration dating.~~

~~f) Preservatives~~

~~Antibiotics are added during production, generally gentamicin sulphate or neomycin not to exceed 30 µg/ml.~~

~~g) Precautions (hazards)~~

~~Vaccination is only recommended for horses in WN positive areas. Vaccinated horses may develop a serological titre that may interfere with the ability to export the horse.~~

~~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 West Nile fever
(see Table in Part 4 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list:
http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/ http://www.oie.int/).
Please contact the OIE Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for West Nile fever

SECTION 2.2.

APINAE

INTRODUCTORY NOTE ON BEE DISEASES

Bees are insects that are closely related to ants and wasps. There are many thousands of species of bee, most of which are not social ~~insects, living,~~ but solitary ~~lives~~ insects. The honey bee, Apis *species, lives as a colony, which is a family of social insects. A honey bee colony is a super-organism with important implications for disease epidemiology, where disease transmission at both individual and colony levels needs to be considered. There are many species and subspecies~~, races and subraces~~ of honey bees that are adapted to their environment.*

Two species are important for bee keeping – the western honey bee Apis mellifera*, and the eastern honey bee* A. cerana*. The Western honey bee is native to the continents of Europe and Africa and is the largest of the cavity-nesting honey bees. It is found in almost every country in the world. ~~There are~~ 24 ~~races~~ subspecies of* A. mellifera *are currently recognised. At least two subspecies of* A. mellifera *are of concern to managed beekeeping. The African bee,* A.m. scutellata*, was accidentally introduced into South America and is known for its defensive behaviour. The Cape bee,* A.m. capensis*, can be a major problem to other ~~races~~ subspecies of* A. mellifera *as it is a serious social parasite of these ~~races~~ in a commercial beekeeping context. ~~The Africanised bee, which is found in South and Central America and some states of the United States of America, is a cross between two subspecies of the western honey bee, the European bees and the South African bee. Apis cerana is important in South and South-East Asia. The colonies are small and docile, but the honey yields are low. In a suitable climate, the western honey bee, A. mellifera, is sometimes preferred for its greater honey production.~~*

It is thought that all bees are susceptible to the known diseases of bees, but different ~~races~~ subspecies may have varying susceptibility. ~~For example, A. cerana is less susceptible to varroosis.~~ The diagnosis and control of honey bee diseases at the colony level is quite difficult. More than with other animals, the possibilities and the methods for clinical observation and diagnosis applied depend on seasonal conditions. This is mainly aggravated in regions with a reduced rearing of brood at certain times of the year, normally in winter, and the temporal production of bee products. In terms of treatment with medicinal products ~~medication~~ and the application of chemical disinfection methods, honey production should always be taken into account, as such treatments can contaminate bee products such as honey, wax and pollen.

When sampling a colony of bees for diagnosis of diseases, ~~live bees~~ sampling of dead bees, if present, in or outside the hive, might best reflect the health status of the colony. If live bees are to be sampled, these must first be killed with diethyl ether or in a deep freezer (–20°C) overnight. Bees may also be killed by submersion in 70% ethyl alcohol, e.g., when collected for diagnosis of acariosis (Acarapis)*. Larval and pupal smears must be made when testing for brood diseases or a piece of comb containing brood showing visible signs of disease may be sent to the laboratory. Honey bees are susceptible to diseases caused by parasites, fungi, bacteria and viruses. Honey bee colonies may also be affected by various pests, predators and adverse environmental factors. ~~Because many honey bee diseases have a cosmopolitan distribution and others do not have a significant impact on bee health and do not produce significant economic damage, at the moment, the~~ Terrestrial Code ~~only considers six pests and diseases of bees.~~*

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CHAPTER 2.2.4.

NOSEMOSIS OF HONEY BEES

SUMMARY

To date, two microsporidian parasites have been described from honey bees: Nosema apis (*Zander*) *and* N. ceranae. Nosema apis *is a parasite of the European honey bee* (Apis mellifera), *and* N. ceranae *of the Asian ~~honey bee~~* (Apis cerana) *and the European honey bees. Both parasites are cross-infective between host species.* Nosema ceranae *has recently been detected in several geographically separated populations of European honey bees in Europe*, *South and North America and Asia*. *The pathological consequences of* N. ceranae *in* Apis mellifera *are not well known. ~~In the following chapter, only~~* Nosema apis *~~is described.~~ Both types are presumed to be very similar, but* N. ceranae *seems to be more sensitive to low temperatures and to be able to reproduce even in high temperatures.* Nosema apis *and* N. ceranae *~~is a parasite that~~ invade the epithelial cells of the ventriculus of the adult honey bee. Infections are acquired by the uptake of spores during feeding or grooming. The disease occurs throughout the world, but treatment of bees can help to prevent the spread of infection to unaffected bee colonies.*

~~The parasite invades the posterior region of the ventriculus, giving rise to large numbers of spores within a short period of time. The parasite is ubiquitous.~~ Nosema *levels generally increase when bees are confined, such as in the autumn and winter in colder climates ~~when the amount of brood is decreasing and perhaps in the early spring when there is an increase in the brood~~. The disease is transmitted among bees via the ingestion of contaminated comb material and water, and by trophallaxis; honey stores and crushed infected bees may also play a role in disease transmission. Spores are expelled with the faeces ~~where they may retain their viability for more than 1 year. Spores may also remain infective after immersion in honey and in the cadavers of infected bees; however they may lose viability after 3 days when submerged in honey at hive temperature~~. The relative importance of faeces, honey and cadavers as reservoirs of infective spores is not fully understood. ~~However, it seems likely that faecal contamination of wax, especially in combs used for brood rearing, or other hive interior surfaces, provides sufficient inoculum for~~ N. apis ~~to be successfully transmitted to the next generation of bees.~~ The spores of N. apis are inactivated by acetic acid or by heating to 60°C for 15 minutes. To be effective, these treatments~~, which inactivate spores on hive surfaces and combs,~~ can be combined with feeding colonies with the antibiotic fumagillin to suppress infections in live bees. ~~The EU~~ Many countries prohibit the use of antibiotic ~~fumigation~~ treatments of honey bees ~~(EU 3/01/081)~~.

Identification of the agent: *In some acute cases, brown faecal marks are seen on the comb and the front of the hive, with sick or dead bees in the vicinity of the hive. However, the majority of colonies show no obvious signs of infection, even when the disease is sufficient to cause significant losses in honey production and pollination efficiency. During winter, there may be an increase in bee mortality. In affected bees, the ventriculus, which is normally brown, can be white and very fragile. Microscopic examinations (×400 magnification) of homogenates of the abdominal contents of affected bees will reveal the oval spores of* Nosema ~~apis~~ *spp., which are approximately 5–7 × 3–4 µm with a dark edge* (N. ceranae *is slightly smaller, but species differentiation is difficult using light microscopy, especially as mixed infections occur*). *Their internal contents can be distinguished after staining with Giemsa's stain.* Nosema ~~apis~~ *spp. spores have a distinctive appearance, with a thick unstained wall and a blue-stained featureless interior. The nuclei within the spores are not visible. Staining can help to distinguish ~~N apis~~* Nosema *spp. from other microbes found in bees.*

The appearance of Nosema ~~apis~~ *spp. spores can be confused with yeast cells, fungal spores, fat and calciferous bodies or cysts of* Malpighamoeba mellificae*. The latter are similar in size to* Nosema *spp. spores, being 6–7 µm in diameter, but are completely spherical instead of oval.*

Positive identifications can be made only by observation of typical spores in the ventriculus or faeces. Very mild infections may not be demonstrable. The extent of infection is determined by counting the spores on a microscope grid and calculating the average number of spores per area and estimating from that the number of spores per bee. Identification to species level is difficult by light microscopy; polymerase chain reaction methods are preferred for this purpose.

Serological tests: *There are no applicable serological tests.*

Requirements for vaccines ~~and diagnostic biologicals~~: *~~There~~ No vaccines are ~~no biological products~~ available.*

A. INTRODUCTION

The microsporidia *Nosema apis* (Zander, 1909) and *N. ceranae* (Fries *et al.*, 1996) are ~~protozoan~~ parasites exclusive of the epithelial cells of the ventriculus of adult bees and ~~the disease~~ both parasites occur throughout the world (~~Matheson, 1996~~ Klee *et al.,* 2007). Based on molecular evidence, microsporidia are now included in the cluster Fungi (Adl *et al.,* 2005); thus, taxonomically, microsporidia are highly specialised parasitic fungi. Infection occurs by the ingestion of spores in the feed (Bailey, 1981; Webster, 1993), via trophallaxis (Webster, 1993) or perhaps after grooming of the body hairs (Bulla, 1977; Fries, 1993 ~~Fries, 1988~~; Webster, 1993). ~~To date, two microsporidian parasites have been described from honey bees: *N. apis* (Zander) and *N. ceranae* (Fries). *Nosema apis* is a parasite of the European honey bee (*Apis mellifera*) and *N. ceranae* of the Asian honey bee (*Apis cerana*) (Fries *et al.,* 1996) and the European honey bee. *Nosema ceranae* has recently been detected in several geographically separated populations of European honey bees in Europe (Higes *et al.,* 2006), South and North America (Klee et al., 2007) and Asia (Huang *et al.*, 2005).~~

Both parasites first invade the epithelial cells in the posterior region of the ventriculus. They produce a fully developed infection throughout the epithelium within 2 weeks. The disease is transmitted among bees via the ingestion of contaminated comb material and water, and by trophallaxis; honey stores and crushed infected bees may also play a role in disease transmission. The infection mechanism of microsporidian parasites is based on mechanical injection of a polar filament protruding from the germinating spore. With physical force, the filament penetrates a host cell membrane into the host cell (ventricular epithelial cells for *N. apis* and *N. ceranae*). Through the filament, the infective sporoplasm is entered into the host cell cytoplasm where parasite replication, and later spore production is initiated (Larsson, 1986). ~~The polar tube of the spore is everted and penetrates the peritrophic matrix of the intestine, particularly in the posterior region of the ventriculus. The sporoplasm passes down the tube and enters the cytoplasm of the epithelial cells, where it reproduces.~~ Auto-infections can occur at the same time as new infections. ~~After a short interval~~ Three days post-infection, mature spores start to develop in large quantities for *N. apis*, approximately a day later for *N. ceranae* (Forsgren & Fries, 2010). ~~The parasite is ubiquitous and multiplies at a specific rate throughout the year~~ Both parasites have a temperature-dependent multiplication rate. *Nosema* spp. levels generally increase when bees are exposed to prolonged confinement, which increases the risk of in hive defecation. In the spring the infection levels may increase rapidly as the bees clean the combs for the expanding egg laying of the queen (Bailey, 1955) and a larger proportion of the bees are exposed to brood temperatures, where parasite replication, at least for *N. apis* is optimal (Lotmar, 1943). *Nosema ceranae* may grow better at slightly higher temperatures compared to *N. apis* (Fenoy *et al.,* 2009) ~~confined such as in the autumn and winter in colder climates when the amount of brood is decreasing and perhaps in the early spring when there is an increase in the brood (Webster, 1993; Weiser, 1961). In winter, spores are rarely to be found, or are only found in heavily infected bees~~.

Any inherent natural defence by a bee colony against a heavy infection with the parasite depends on the colony size as well as on the prevailing weather conditions during the early part of the autumn of the previous year (Steche, 1985). If these conditions are unfavourable, the overall life expectancy of the colony is reduced. This may lead to the premature death of bees during winter or early spring. In a typical case of a colony being depleted because of a *Nosema* infection, the queen can be observed surrounded by a few bees, confusedly attending to brood that is already sealed.

In faecal droppings, spores of *N. apis* may retain their viability for more than 1 year (Bailey, 1962). Spores may also remain viable for up to 4 months after immersion in honey (White, 1919) and for up to 4.5 years in the cadavers of infected bees (Steche, 1985). The spores may lose viability after only 3 days when submerged in honey at hive temperature (Morgenthaler, 1939). Faecal contamination of wax, especially in combs used for brood rearing or other hive interior surfaces, provides sufficient inoculum for *N. apis* to be successfully transmitted to the next generation of bees and is probably the primary source of infection (Bailey, 1955). For *N. ceranae,* the durability of spores in different situations remains to be investigated, but they appear to withstand desiccation and heat better than *N.* apis spores (Fenoy *et al.,* 2009) whereas they are more sensitive to freezing temperatures (Forsgren & Fries, 2010). The relative importance of faeces, honey and cadavers as reservoirs of infective spores is not fully understood and it seems that temperature may have a marked effect on the rates at which spores lose viability, regardless of their medium (Morgenthaler, 1939).

Spores of *N. apis* may be killed by heating hive equipment or tools to a temperature of at least 60°C for 15 minutes. Combs may be sterilised by heating to 49°C for 24 hours (Cantwell & Shimanuki, 1970). ~~The corresponding data are lacking for~~ This cannot be used for *N. ceranae,* which can survive up to 60°C (Fenoy *et al*., 2009). Fumes from a solution of at least 60% acetic acid will inactivate spores of *N. apis* within a few hours, depending on the concentration; higher concentrations are even more effective and will kill spores within a few minutes (Bailey, 1957 ~~de Ruiter & van der Steen, 1989~~). The corresponding data are lacking for *N. ceranae*. Such procedures come under the jurisdiction of national control authorities with protocols that vary from country to country. Disinfection can be carried out, for example, by putting acetic acid solution into bowls or on to sponges that can soak up the liquid on top of a sealed stack of boxes with combs. Following disinfection after an outbreak, all combs should be well ventilated prior to use. Suppression of *Nosema* disease can also be achieved by feeding an antibiotic, fumagillin, in sugar syrup to the colony (Cantwell & Shimanuki, 1970). Use of antibiotics for honey bees is forbidden in many countries and in the European Union.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

In acute forms of infection, especially in early spring, brown faecal marks may be noted on the comb and the front of the hive (Bailey, 1967). Lack of seasonal prevalence and symptoms such as faecal deposits have been reported for *N. ceranae* (Higes *et al.,* 2008). At the entrance to the hive, sick and dead bees may be seen, although other causes, such as pesticide poisoning and diseases of adult honey bees (such as acarapidosis should be eliminated first if this is the case. The detection of these infectious diseases requires microscopic examination. During winter, *N. apis*-infected colonies may become severely depleted of bees or die out altogether. The majority of *N. apis*-infected colonies will appear normal, with no obvious signs of disease even when the disease is sufficient to cause significant losses in honey production and pollination efficiency (Anderson & Giacon, 1992; Fries *et al.,* 1996). A proper diagnosis can be made ~~only~~ by microscopic examination of adult bee abdomen or ventriculus, by molecular tools (polymerase chain reaction [PCR]) or by transmission electron microscopy (TEM). To diagnose a *Nosema* ~~apis~~ spp. infection using microscopy the posterior pair of abdominal segments is removed with a forceps to reveal the ventriculus, complete with the malpighian tubules, the small intestine and rectum. The ventriculus is normally brown but, following a *Nosema* spp. infection, it can become white and fragile. However, this appearance is given by other causes of intestinal disturbance, for example feeding on indigestible food stores, such as syrup containing actively growing yeast. For a reliable diagnosis, a number of bees in a sample should be examined. For example, 60 bees examined in a composite sample will detect a 5% infection level with 95% probability.

a) Microscopy

It is necessary to attempt to distinguish between a *Nosema* ~~apis~~ spp. infection and an infection caused by *Malpighamoeba mellificae* (Webster, 1993). There is quite often an indication of dysentery in a *N. apis* infection. In an *M. mellificae* infection, there may be diarrhoea, often of a sulphur-yellow colour and with a distinct odour. Characteristics of *M. mellificae* cysts are described later. Secondary mixed infections may occur (Morgenthaler, 1939). A simple, non-quantitative method for detecting *Nosema* ~~apis~~ spp. infection is as follows: sampled bees should be obtained from the hive entrance in order to avoid sampling ~~individuals~~ young bees that are less likely to be infected. ~~under the age of 8 days, which would lead to 'false negatives' because no spores from the protozoan in question would be determined.~~ At least 60 bees should be collected in order to detect 5% of diseased bees with 95% confidence (Fries, 1993 ~~Fries, 1988~~). Before sending to the laboratory, the bees should be fixed in 4% formol, 70% ethyl alcohol or frozen in a standard freezer in order to prevent them from decomposing and to improve their reception and organisation in the laboratory. The abdomens of the bees to be examined are separated and ground up in ~~2–3~~ 5 ml of water. Then water is added representing a total volume of 1 ml per bee in the sample. A drop of the suspension is placed on a slide under a cover-slip and examined microscopically at ×400 magnification, under bright-field or phase-contrast optics. ~~To quantify the average infection level spore counts in a haemacytometer can be used This is a slight simplification of Cantwell's original method (Cantwell, 1970) or bees can be diagnosed individually to yield the proportion of infected bees~~

The spores are about 5–7 µm long and 3–4 µm wide (*Nosema ceranae* is slightly smaller than *Nosema apis*). They are completely oval with a dark edge. Their contents, consisting of nucleus, sporoplasm and polar tube, cannot be seen. Dyes are usually not necessary. *Nosema* spp. spores must be differentiated from yeast cells, fungal spores, fat and calciferous bodies, and from *M. mellificae* cysts, which are spherical and approximately 6–7 µm in diameter.

When air-dried, ethanol-fixed smears of infected tissue are stained with Giemsa's stain (10% in 0.02 M phosphate buffer) for 45 minutes. *Nosema* ~~apis~~ spp. spores will have a distinctive appearance, with thick unstained walls and an indistinct blue interior, without visible nuclei. Insect cells, fungal spores and other protozoa stained in this way will generally have thinner walls, blue/purple cytoplasm and magenta-coloured nuclei.

To quantify the average infection level, spore counts in a haemacytometer can be used (Cantwell, 1970) or bees can be diagnosed individually to yield the proportion of infected bees. ~~In order to obtain accurate, reliable and meaningful quantification of levels of *Nosema* spp. infections in honey bees, a~~ A standardised procedure such as the following must be used.

A sample of older worker honey bees is taken from the hive entrance or from peripheral frames if weather does not permit flight conditions. The abdomens of ~~from 10 to 50~~ 60 individuals are macerated in 5 ml of water using a mortar and pestle and 50 ml of water is added for a total volume of 1 ml per bee (5 ml is added later). When tissue pieces have become quite fine, the suspension is filtered through two layers of muslin (thin loosely woven cotton fabric) in a funnel leading to a graduated centrifuge tube. A second 5 ml of water is used to rinse the pestle, swirl around the inside of the mortar and pour through the subsample in the funnel. ~~Water levels are equalised in the tubes and the suspensions are centrifuged for 6 minutes at 800 g. The supernatants are decanted and the tubes are refilled to the 10 ml level. Using disposable pipettes and a rubber bulb, the pellets are resuspended by repeated uptake and forcible ejection through the pipette tips.~~ When the suspension appears to be homogenous after shaking, a sample is taken to fill the calibrated volume under the cover-slip of a haemocytometer (blood cell counting chamber). After a few minutes the spores will have settled to the bottom of the chamber. *Nosema* spp. spores appear transparent but with a very distinct dark edge and are 5–7 µm long and 3–4 µm wide. They are best seen using a magnification of ×400 and bright-field or phase-contrast optics. The number of spores in each square is counted. Where a spore lies over the edge of a square, count only those spores that straddle the left and upper edges of the square, not the right and bottom edges. The size of these chambers can vary with manufacturer but they mostly consist of two separate chambers, each with a defined volume ($0.1 mm^3$) containing a marked counting grid with an area of 1 mm^2. The whole grid consist of 3 × 3 large squares, separated by triple lines. Each large square is further subdivided into 16 smaller squares subdivided by double lines, in total 144 squares. The spores are counted in the smaller squares with the area of 1/25 mm^2. When the counting is completed, the number of spores per bee in the sample can be calculated according to the formula:

$$Z = \alpha/\beta \times \delta \times 250{,}000$$

Where

Z = spore numbers per bee
α = total number of spores counted
β = number of squares counted
δ = dilution factor

The number 250,000 is used because the volume in each counted square is 1/250 000 ml and the equation uses the average number of spores per counted square. ~~One *Nosema apis* spore, observed in the haemocytometer's entire central square millimetre grid (25 × 16 = 400 small squares), is equal to an average of 10.000 spores per bee.~~ If no spores are seen, the result should be designated 'not detected', but that does not mean that the bees are not infected. Regulatory agencies will decide on the level of infection useful for their purposes.

A laboratory method for the simultaneous detection of *Nosema* spp. spores and *M. mellificae* cysts consists of the individual examination of the colonies using ~~30–~~60 bees per colony. A suspension of the abdomens of dead bees is prepared by grinding with 5–10 ml water; the volume of water depending on the number and condition of the bees. The suspension must be filtered to remove debris that would interfere with the examination, first through a 100 µm and then a 40 µm filter. Parts of the malpighian tubules pass through the 100 µm filter, but are collected on the 40 µm filter. They are placed on a slide or bacterial counting chamber and examined at ×400 magnification. Only a few tubules are filled with cysts after an *M. mellificae* infection. The normal structure of malpighian tubules is not visible in this case. Only cysts inside the malpighian tubules can be taken as a positive result, because *M. mellificae* cysts are often confused with fungal spores and yeast cells.

b) Culture

~~There are no cultural methods for growing these organisms.~~

Several lepidopteran cell lines have been shown to be susceptible to both *N. apis* and *N. ceranae* infection. Susceptibility was recently demonstrated for the following cell lines (Gisder *et al.,* 2010): MB-L2 (*Mamestra brassicae*), Sf-158 and Sf-21 (*Spodoptera frugiperda*), SPC-BM-36 (*Bombyx mori*), IPL-LD-65Y (*Lymantria dispar*), and BTI-Tn-5B1-4 (*Trichoplusia ni*). All these cell lines can be obtained through national cell culture collections together with protocols on how to maintain and passage the cell lines. However, the available protocols do not yet allow the continuous propagation of *Nosema* spp. in cell culture. Thus, it is not yet possible to replace infection of bees for the production of spore suspensions.

c) Polymerase chain reaction (PCR)

Different methods have been developed to distinguish *N. apis* from *N. ceranae*. A multiplex PCR is described below with which both pathogen types along with *Nosema bombi* can be clearly identified at the same time (~~Martin-Hernandez *et al.*, 2007~~ Fries *et al.*, 2013).

o Sample preparation for PCR

~~The abdomens of 10–20 adult honey bees from each sample are macerated in 10 ml distilled water (PCR grade) and the suspension is then filtered and centrifuged at 800 ***g*** for 6 minutes. For DNA extraction, spore germination is induced with 200 µl freshly prepared germination buffer (0.5 M sodium chloride, 0.5 M sodium hydrogen carbonate, pH to 6.0 with ortophosphoric acid), and the mixture is incubated at 37°C for 15 minutes. The DNA extraction can be easily carried out using routine procedures or commercial kits, such as High Pure PCR Template Preparation Kit (No. 1796828 Roche Diagnostic).~~

Place a maximum of 30 bees in a filter grinding bag. Add 0.5 ml (DNAase/RNAase free) ddH_2O per bee and homogenise the mixture using a homogeniser. Flash-freezing in liquid nitrogen is possible prior to homogenisation to aid in mechanically breaking open cells. Without access to a robot, a pestle can be used to crush the bee tissue (frozen tissue if flash-frozen) to generate a homogeneous homogenate. Transfer 100 µl of the liquid homogenate into a microcentrifuge tube and centrifuge for 3 minutes at 16,100 ***g*** to precipitate the microsporidia and other cellular material. Discard the supernatant. Freeze the pellet by using liquid nitrogen and crush using a pestle until pulverized (in order to break open *Nosema* spore walls), and repeat 2–3 times so that *Nosema* DNA goes into solution. The DNA extraction can be easily carried out using routine procedures or commercial kits. Complete the final elution step in 100 µl AE buffer.

o Multiplex PCR

~~With this technique both microsporidians (*N. apis* and *N. ceranae*) can be distinguished in just one PCR because of the use of specific primers with no interference. PCR reactions are performed in 50-µl volumes containing 5 µl of template DNA, 25 µl of High Fidelity PCR master mixture (catalogue no. 12140314001; Roche Diagnostic), 0.4 µM of each primer, 0.4 mM of each deoxynucleoside triphosphate, 3 mM Cl_2Mg, 0.2 mg/ml bovine serum albumin, 0.1% Triton X-100, and 5 µl of *N. apis* or *N. ceranae* DNA template. The parameters for amplification are: an initial PCR activation step of 2 minutes at 94°C, followed by 10 cycles of 15 seconds at 94°C, 30 seconds at 61.8°C, and 45 seconds at 72°C, and 20 cycles of 15 seconds at 94°C, 30 seconds at 61.8°C, and 50 seconds at 72°C plus a 5 second elongation cycle for each successive cycle and a final extension step at 72°C for 7 minutes. Negative controls (from DNA extraction) are included in all PCR experiments.~~

~~The molecular weights of PCR products are determined by electrophoresis in a 2% agarose TAE (Tris-acetate-ethylene diamine tetra-acetic acid) gel in standard TAE buffer, stained with ethidium bromide, and visualised using UV illumination.~~

~~Primers selected for detection of *N. ceranae* and *N. apis* in multiplex PCR:~~

~~Primer~~	~~Sequence[a]~~	~~PCR product size (bp)~~	~~Specificity~~
~~218MITOC FOR~~	~~5' CGGCGACGATGTGATATGAAA ATATTAA 3'~~	~~218–219[b]~~	~~*N. ceranae*~~
~~218MITOC REV~~	~~5' CCCGGTCATTCTCAAACAAAA AACCG 3'~~		
~~321APIS FOR~~	~~5' GGGGGCATGTCTTTGACGTACTATGTA 3'~~	~~321~~	~~*N. apis*~~
~~321APIS REV~~	~~5' GGGGGGCGTTTAAAATGTGAAACAACTATG 3'~~		

~~[a]CG tails added to primers are underlined.~~

~~[b]There is a 1-bp difference in the *N. ceranae* amplicon size depending on the sequences for *N. ceranae* available in GenBank (http://www.ncbi.nlm.nih.gov).~~

For multiplex PCR amplification of partial 16S rRNA (=SSU rRNA) gene fragments, the following primer combination can be used. Primers were designed based on alignment of all available sequence data in GeneBank of the 16S rRNA gene from *N. apis, N. bombi* and *N. ceranae*.

Mnceranae-F forward primer: 5'-CGT-TAA-AGT-GTA-GAT-AAG-ATG-TT-3'
Mnapis-F forward primer: 5'-GCA-TGT-CTT-TGA-CGT-ACT-ATG-3'
Mnbombi-F forward primer: 5'-TTT-ATT-TTA-TGT-RYA-CMG-CAG-3'

Muniv-R: reverse primer: 5'-GAC-TTA-GTA-GCC-GTC-TCT-C-3'

Note that the Mnbombi-F primer contains variable sites to account for the sequence diversity observed for this species.

PCR product size:

for *N. ceranae:* 143 bp
for *N. bombi:* 171 bp
for *N. apis:* 224 bp

PCR conditions:

1 µl of DNA (ca. 1 ng)
0.5 U of Taq polymerase
2× Taq reaction buffer (3 mM $MgCl_2$)
0.3 mM of each dNTP (dNTP mix)
0.4 µM of Mnceranae F
0.4 µM of MnapisF
0.5 µM of Mnbombi-F
0.5 µM of Muniv-R
in 10 µl total volume

Amplification is carried out in a thermocycler under the following conditions: Initial denaturation step of 95°C for 2 minutes, 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 60 seconds, with a final extension step of 72°C for 5 minutes. Visualisation of the amplification products is made using standard procedures.

2. Serological tests

There are no serological tests available.

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

No biological products are available.

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NB: There are OIE Reference Laboratories for bee diseases
(see Table in Part 4 of this *Terrestrial Manual* or consult the OIE web site for the most up-to-date list:
http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/ http://www.oie.int/).
Please contact the OIE Reference Laboratories for any further information on
diagnostic tests and reagents for bee diseases

CHAPTER 2.2.5.

SMALL HIVE BEETLE INFESTATION
(*Aethina tumida*)

SUMMARY

The small hive beetle, Aethina tumida *Murray 1867* (*Coleoptera: Nitidulidae*), *is a parasite and scavenger of honey bee colonies. Adults and larvae small hive beetles feed on honey bee brood, honey and pollen, thus causing brood death,* fermentation *brood, fermenting of honey and comb destruction. often resulting in the full The beetles can promote structural collapse of the nest and absconding of the colony cause the adult honey bees to abscond. The extent of beetle-associated damage likely depends on climate conditions, among other factors. Small hive beetles tend to be more problematic in areas with warm temperatures and high humidity than in areas with lower temperatures and humidity. The small hive beetle can be a serious problem in honey-extracting facilities where stored comb, honey and wax cappings are potential feeding and breeding areas. Development Beetle development from egg to adult requires 3–12 weeks, depending on humidity, temperature and food availability. The flying adult beetles actively infest honey bee colonies of all strengths and sizes.*

Identification of the agent: *An infestation by Aethina tumida the small hive beetle can be recognised either indirectly via colony-wide damage associated with the beetle or directly via eggs, larvae and adults. An early diagnosis can be made after opening the colony and finding adult beetles under the colony lid, on the bottom board, or hiding in the combs (especially peripheral combs). Intra- and extra-colonial acaricides and insecticides presently are used to kill the adult beetles and larvae while intra- and extra-colonial traps can be used to find the beetles.*

Serological tests: *No serological tests are applicable.*

Requirements for vaccines and diagnostic biologicals: *No biological products vaccines are available.*

A. INTRODUCTION

The small hive beetle (hereafter referred to as "beetle" or "small hive beetle"), *Aethina tumida* Murray, Coleoptera: Nitidulidae (Murray, 1867), is native to sub-Saharan Africa (Hepburn & Radloff, 1998) but has been introduced to found in the United States of America (1996), Egypt (2000) and Australia (2002) (Neumann & Ellis, 2008 Neumann & Elzen, 2004). It was introduced Introductions were found in different regions of to Canada in 2002, but did not establish; it was reintroduced 2006, and annually from 2008 to 2012. 2009, and 2010 However only the population in southern Ontario appears to be established (Kozak, 2010) and it has not been determined though it remains unclear if the beetle can establish populations there it is established. *Aethina tumida* can be spread by active flying, migratory beekeepers, or transportation of infested hive products (Hood, 2004; Neumann & Elzen, 2004). Larvae and eggs of the small hive beetle have been identified in cages of imported queens in Portugal (2004), but all bee hives were destroyed immediately (Neumann & Ellis, 2008). The small hive beetle can be spread by active flying, movement of infested honey bee colonies, or transportation of infested hive products (Lundie, 1940; Hood, 2004). Within its native range in Africa, the beetle is usually considered a minor pest, and reproduction appears more successful in weak and stressed colonies or in recently abandoned nests (Neumann & Elzen, 2004) (Lundie, 1940). However, within its new ranges it can cause considerable damage in colonies of European honey bee subspecies within its new ranges (Ellis, 2004; Elzen *et al.*, 1999; Hood, 2004; Neumann & Elzen, 2004).

1. Life cycle

The infesting *A. tumida* females small hive beetle adults mate in the colony (more than 1000 adult beetles may occur within a colony [Elzen *et al.*, 1999]) and the female beetles oviposit several eggs in typical clutches in small

cracks~~, in cells~~ or within capped brood cells (Ellis, 2005; ~~Ellis *et al.*, 2003c;~~ Lundie, 1940; ~~Neumann & Elzen; 2004~~). More than 1000 adult beetles may occur within a colony in some situations (Elzen *et al.*, 1999). Adult beetles can survive up to 6 months and females can oviposit about 1000 eggs in their lifetime (Lundie, 1940), though Hood (2004) suggested the upper limit may be 2000 eggs. Successful egg emergence is negatively correlated with relative humidity, with fewer eggs hatching at a relative humidity <50%. The larvae ~~hatch~~ emerge from the eggs after 1–6 days (most within 3 days) and feed on pollen, honey and bee brood like the adults (Lundie, 1940; ~~Neumann & Elzen; 2004;~~ Schmolke, 1974). Adult beetles also can be fed by worker bees via trophallaxis, especially while confined in bee-guarded "prisons" ~~(Ellis *et al.*, 2002~~ Ellis, 2005). Larval development takes about 8–29 days ~~(~~depending on food availability and temperature (~~Ellis *et al.*, 2002;~~ Ellis *et al.*, 2002b Lundie, 1940; ~~Neumann & Elzen; 2004;~~ Schmolke, 1974). Following this, the larvae reach the wandering phase ~~(Lundie, 1940)~~ and leave the colony to pupate ~~for pupation~~ in soil, ~~mostly in close proximity to the hive (Pettis & Shimanuki, 2000)~~ surrounding the colony (Lundie, 1940). Pupation takes about 2–12 weeks depending on temperature and soil moisture (Ellis et al., 2004~~a~~). Emerging adults leave the soil and can ~~actively fly over long distances (>10 km)~~ fly to search for new host colonies, thereby completing the life cycle of ~~*A. tumida*~~ the small hive beetle.

2. Impact of the disease

The reason~~s~~ for the apparent difference in the impact of the small hive beetle on colonies within its native range ~~compared with~~ and those in its new ranges are not well understood (~~Ellis, 2004~~ Ellis & Hepburn, 2006). They may include quantitative behavioural differences between African and European honey bee subspecies, different beekeeping techniques ~~and~~ used, climatic differences, and/or escape from natural enemies, among other plausible hypotheses (~~Ellis, 2004;~~ Hood, 2004; Neumann & Elzen, 2004).

~~Adult beetles can survive up to 6 months and females can oviposit about 1000 eggs in their life time (Lundie, 1940).~~

While bee colony damage due to adult~~s~~ beetles is relatively minor, ~~it nevertheless~~ the adults can cause ~~absconding of~~ colonies to abscond (i.e. the ~~colony~~ adults bees completely abandon the nest, Ellis *et al.*, 2003~~b~~). If not prevented by the bees ~~(Ellis *et al.*, 2003c; Neumann & Härtel, 2004)~~, larval ~~growth (several hundreds or thousands of individuals)~~ feeding behaviour often is ~~usually~~ associated with fermentation of ~~the~~ stored honey, causes severe damage to combs and often results in the full structural collapse of the nest (~~Hepburn & Radloff, 1998~~ Lundie, 1940). Economic losses also can be associated with beetle infestations in the honey-extracting facility. Environmental condition~~s~~ generally associated with extracting facilities, such as high temperature and humidity, provide optimal conditions for beetle development. Cryptic low-level reproduction may also occur either in the debris or underneath hive inserts without any signs of ~~damage to the beekeeper (Schmolke, 1974)~~ colony damage (Spiewok & Neumann, 2006).

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

a) Adult beetles

The first sign of an infestation by ~~*A. tumida*~~ the small hive beetle is the occurrence of adult beetles (Figure 1). Adult beetles are ~5 mm long and ~3 mm wide, with females being slightly longer than males (Ellis *et al.*, ~~2004b~~ 2001). The adults are a dark brown to black colour (lighter shortly after eclosion) ~~in the colony (Fig. 1)~~. During inspections, adults avoid sunlight, hide, and can be observed while running for cover into corners or ~~in a typical fashion~~ similarly over the combs. Adults can be confused with other nitidulid beetles, ~~from the same family~~ which can also be associated with colonies (~~e.g. *Cychramus luteus*~~ see Neumann & Ritter, 2004 and Ellis *et al.*, 2008 for examples).

Fig. 1. *Dorsal (left) and ventral (right) view of an adult small hive beetle. Photographs by Lyle Buss (left) and Josephine Ratikan (right), University of Florida.*

b) Beetle eggs, larvae and pupae

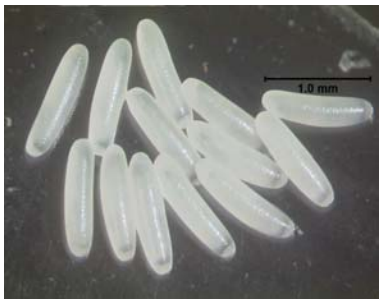

Fig. 2. *Small hive beetle eggs. Photograph by Josephine Ratikan, University of Florida.*

~~**a) Beetle eggs, larvae and pupae**~~

Small hive beetle eggs (Figure 2) are white, ~~and bean shaped~~ ~1.4 × 0.26 mm (length × width); ~2/3 of the size of a honey bee egg~~)~~, and are oviposited in clutches ~~(up to 210)~~ in cracks, on the bottom board, on the combs and underneath the cappings of sealed brood (~~Ellis *et al.*, 2003c)~~ cells. Larvae (Figure 3) are whitish, ~~often covered with a slimy sticky coating,~~ up to ~1~~.2~~ cm long (wandering phase), have three pairs of legs, and have dorsal spikes. Larvae can be found mining in the wax combs (Lundie, 1940) or in colony debris (Spiewok & Neumann, 2006). Larval infestations are typically associated with a rotten smell ~~(e.g. rotting orange)~~ due to death of honey bee brood and/or fermentation of the stored honey. Wandering larvae often leave smear trails (or "slime") inside and outside the colony ~~Such wandering larvae and pupae (whitish, ~5 mm long and 3 mm wide) can be found~~ (Figure 4). Once in the ground, the larvae excavate small pupation chambers (Figure 5) 1–20 cm deep in the soil ~~usually in close proximity to colonies (<180 cm,~~ (Pettis & Shimanuki, 2000), develop into pupae (Figure 6, whitish to dark brown depending on age, ~5 mm long and 3 mm wide) and then into adults. Most larvae tunnel into soil that is <180 cm from the colony (Pettis & Shimanuki, 2000).

Fig. 3. *Dorsal (left) and ventral (right) view of a small hive beetle larva. Photographs by Josephine Ratikan, University of Florida.*

Fig. 4. *Comb damage attributed to the feeding/crawling habits of small hive beetle larvae. Notice the ''slime'' on the frame (i.e. the wax comb looks ''wet'' and it ''glistens''). This is caused by the fermentation of honey, which is moved around the comb by*

Fig. 5. *Small hive beetle larvae that has tunnelled into the soil and hollowed out a chamber in which to pupate. Photograph credit, University of Georgia.*

crawling larvae. Beetle larvae can be seen in cells in the centre of the comb, where brood was present originally. Photograph credit, University of Georgia.

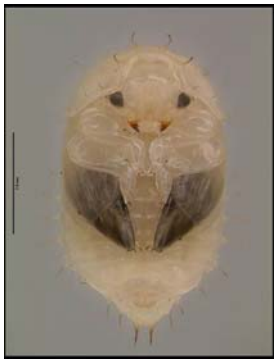

Fig. 6. *Small hive beetle pupa (ventral view). Photograph by Lyle Buss, University of Florida.*

It is difficult to find beetle eggs in a colony, especially at low levels of infestation. However, one can look in cracks/crevices around the nest or in capped brood cells that have small holes in the cappings, possibly indicating that a female beetle has punctured the capping and oviposited within the cell. Small hive beetle pupae can be found by sifting the soil around the colony and looking for the pupal chambers or the pupae themselves.

c) Manual colony examination

When monitoring honey bee colonies for the presence of ~~*A. tumida*~~ small hive beetles, an examination of the hive may provide an early indication of infestation. Adult beetles can be observed hiding inside the comb cells and in the debris at the bottom of the colony. ~~Colony examinations start by removing the hive roof and placing it upside down on the ground next to the hive. Remove the hive chamber, i.e. supers and upper brood chamber (in double brood chamber colonies) and place them on the upturned roof for a few minutes. Place the hive crown board on top. A few minutes later lift the boxes out of the way and scan for beetles on the inner surface of the upturned roof. Then frames are screened one by one for the presence of adults, larvae and eggs. During cool weather, adults tend to stay close to or within the bee cluster. In warmer periods beetles are found more often on the bottom board or the outer-most frames.~~ Detailed instructions for colony examination follow and are modified from Ellis *et al.* (2002a) and Ellis & Delaplane (2006). This method can be used to search for beetle adults and larvae, if larval infestations are moderate to high.

Fig. 7. *Inspecting a colony for adult small hive beetles. The individual on the right has shaken the bees onto a piece of plywood. He followed this by bouncing both faces of the framed comb onto the wood (dislodging beetles in the cells). The individual on the left is shifting through the adult bees and using a mouth aspirator to collect the beetles. Photograph by Keith Delaplane, University of Georgia.*

Notes:

- This procedure is best accomplished with two people, one to work the colony and the second to collect the beetles if quantification is desired. Only one person is needed if beetle qualification is the sole desired outcome.
- Some beetles inevitably fly away or hide from view during this procedure. The number of beetles that escapes is presumed to be low (<5%).
- This procedure is best used for qualification of adult beetles. However, larval beetles can be found this way as well.

i) Place a sheet of opaque plastic (~2 × 2 m, preferably white or light in colour) or plywood in front of the colony which you want to inspect for beetles.

ii) Lightly smoke the colony.

iii) Remove the lid from the colony and bounce the lid on the plywood. This should be done to dislodge all adult bees and beetles adhering to the lid.

iv) A second individual (the beetle collector) should comb through the bees (this can be done with the hand or using a small stick) and collect all adult beetles seen using an aspirator. All bees on the plywood should be inspected since beetles easily can be concealed by clusters of bees (Figure 7).

v) Remove the outermost frame in the uppermost super (i.e. the uppermost "box" containing bees) and shake the bees from the frame onto the plywood.

vi) The beetle collector should repeat step iv.

vii) Once the bees have been shaken from the frame, the frame should be turned onto its face and bounced against the plywood to dislodge adult beetles from the comb. This step should be repeated two-to-three times for both sides of the frame.

viii) The beetle collector should repeat step iv.

ix) The individual working the colony should repeat step vii to all frames in the uppermost super and then bounce the empty super on the plywood. This step should be repeated for all supers, all frames, and the bottom board of the colony.

d) Colony examination using inserts and traps

Less labour intensive diagnosis is feasible using hive inserts. Such inserts have holes that allow for the beetles to hide in the corrugations but prevent bees from entering. They can be placed on the bottom board of the colony. To detect the beetles, place a piece of corrugated cardboard or similar material (15 cm × 15 cm), with one surface peeled away to expose the ridges, on the bottom board of the bee hive with the ridged side down. Cover it with wood to fit underneath the frames on the bottom board. Leave the insert in the colony for ≤ 3 days, remove it and examine for adult and larval beetles. A similar plastic insert (Figure 8) has been shown to detect adult beetles at the apiary level and can be predictive of adult beetle infestation rates (Schaefer *et al.,* 2008). Various insecticides can be used to kill adults in these inserts.

Fig. 8. *Plastic insert used to detect adult small hive beetles. The plastic insert contains small holes (left) in which adult beetles hide when inserted onto the bottom board of a colony, through the colony entrance (right). The insert must be used in conjunction with a traditional solid bottom board rather than a screened bottom board. Photographs by James Ellis (left) and Stephanie Kimball (right), University of Florida.*

Similarly, any number of commercially available small hive beetle traps can be inserted into colonies in accordance with the manufacturers' instructions. No specific trap recommendations are made here as there are many designs and most have similar efficacies. The majority of small hive beetle traps are placed on the bottom of the colony, within a frame of a colony, or between the upper part (or top bar) of two frames in a

colony. Apple cider vinegar typically is added to the traps as it acts as an adult beetle attractant. Additionally, mineral or vegetable oil should be added to the traps as a killing agent. The adult beetles will wander into the trap, thus facilitating their qualification. The traps can be used in colonies to monitor for the presence of adult beetles regularly.

2. Serological tests

No serological tests are available for routine laboratory diagnosis.

~~3. Treatment~~

~~In countries with infestations of *A. tumida*, control has focused on chemical treatments with acaricides and insecticides (Baxter *et al*., 1999a; 1999b; Elzen *et al*., 1999). Acaricides, non toxic to bees, are used in traps intra-colonially (Elzen *et al*., 1999) to kill the adults. Similarly, insecticides are used as a ground drench to kill wandering larvae and pupae (Baxter *et al*., 1999a; 1999b). Such treatments carry the risks of both pest resistance and residues in the hive products (Neumann & Elzen, 2004). Alternative, more sustainable controls are moderately efficient so far (Ellis, 2004; Hood, 2004; Hood & Miller, 2005; Neumann & Elzen, 2004) and currently under investigation (Ellis, 2004; Ellis *et al*., 2001; 2003a; Hood, 2004; Hood & Miller, 2005; Muerrle *et al*., 2006).~~

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

There are no biological products available.

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FURTHER READING

An FAO publication, Honey bee diseases and pests: a practical guide, W. Ritter & P. Akratanakul (eds). Agricultural and Food Engineering Technical Report No. 4. FAO, Rome, Italy, 42 pp. ISSN 1814-1137 TC/D/A0849/E, is available free of charge at:
http://www.fao.org/WAICENT/faoINFO/AGRICULT/ags/subjects/en/industFoodAg/pdf/AGST_techrep_4.pdf

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NB: There are OIE Reference Laboratories for bee diseases
(see Table in Part 4 of this *Terrestrial Manual* or consult the OIE web site for the most up-to-date list:
http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/ http://www.oie.int).
Please contact the OIE Reference Laboratories for any further information on
diagnostic tests and reagents for bee diseases

CHAPTER 2.3.2.

AVIAN INFECTIOUS BRONCHITIS

SUMMARY

Avian infectious bronchitis (*IB*) *is caused by the gammacoronavirus infectious bronchitis virus* (*IBV*). *The virus causes infections mainly in chickens and is a significant pathogen of commercial meat and egg type birds. IB is an acute, contagious disease characterised primarily by respiratory signs in growing chickens. In hens, decreased egg production and quality are often observed. Some strains of the virus are nephropathogenic and produce interstitial nephritis and mortality. The severity of IBV-induced respiratory disease is enhanced by the presence of ~~bacterial~~ other pathogens, including bacteria, leading to chronic complicated airsacculitis. Diagnosis of IB requires virus isolation or demonstration of viral nucleic acid from diseased flocks. Demonstration of a rising serum antibody response may also be useful. The widespread use of live and inactivated vaccines may complicate both the interpretation of virus isolation and serology findings. The occurrence of antigenic variant strains may overcome immunity induced by vaccination.*

Diagnosis requires laboratory testing. Virus ~~isolation~~ detection and identification is preferred. Reverse-transcription polymerase chain reaction (*RT-PCR*) *techniques are commonly used to identify the IBV genotype. Haemagglutination inhibition* (*HI*) *tests to determine serotype* (*appropriate in young birds ~~only~~*) *and enzyme-linked immunosorbent assays* (*ELISA*) *are often used for sero-diagnosis and/or monitoring. Supplementary tests include electron microscopy, the use of monoclonal antibodies, virus neutralisation* (*VN*), *immunohistochemical or immunofluorescence tests, and immunisation-challenge trials in chickens.*

Identification of the agent: *For the common respiratory form, IBV is most successfully isolated from tracheal mucosa and lung several days to one week following infection. For other forms of IB, kidney, oviduct, the caecal tonsils of the intestinal tract or proventriculus tissues are better sources of virus depending on the pathogenesis of the disease.*

Specific pathogen free chicken embryonated eggs or chicken tracheal organ cultures (*TOCs*) *from embryos may be used for virus isolation. Following inoculation of the allantoic cavity, IBV produces embryo stunting, curling, clubbing of the down, or urate deposits in the mesonephros of the kidney, often within three serial passages. Isolation in TOCs has the advantage that IBV produces stasis of the tracheal cilia on initial inoculation. RT-PCR is increasingly being used to identify the spike* (*S*) *glycoprotein genotype of IBV field strains. Genotyping using primers specific for the S1 subunit of the S gene or sequencing of the same gene generally provides similar but not always identical findings to HI or VN serotyping. Alternatively, VN or HI tests using specific antiserum may be used to identify the serotype.*

Serological tests: *Commercial ELISA kits may be used for monitoring serum antibody responses. The antigens used in the kits are broadly cross-reactive among serotypes and allow for general serological monitoring of vaccinal responses and field challenges. The HI test is used for identifying serotype-specific responses to vaccination and field challenges especially in young growing chickens. Because of multiple infections and vaccinations, the sera of breeders and layers contain cross-reactive antibodies and the results of HI testing cannot be used with a high degree of confidence.*

Requirements for vaccines ~~and diagnostic biologicals~~: *Both live attenuated and oil emulsion inactivated vaccines are available. Live vaccines, attenuated by serial passage in chicken embryos or by thermal heat treatment, confer better local immunity of the respiratory tract than inactivated vaccines. The use of live vaccines carries a risk of residual pathogenicity associated with vaccine back-passage in flocks. However, proper mass application will generally result in safe application of live vaccines.*

Inactivated vaccines are injected and a single inoculation does not confer protection unless preceded by one or more live IBV priming vaccinations. Both types of vaccines are available in combination with Newcastle disease vaccine; in some countries inactivated multivalent vaccines are available that include two to three IBV antigens or Newcastle disease, infectious bursal disease, reovirus and egg-drop syndrome 76 viral antigens.

A. INTRODUCTION

Avian infectious bronchitis (IB) was first described in the United States of America (USA) in the 1930s as an acute respiratory disease mainly of young chickens. A viral aetiology was established, and the agent was termed avian infectious bronchitis virus (IBV). The virus is a member of the genus *Gammacoronavirus*, subfamily *Coronavirinae*, family *Coronaviridae*, in the order Nidovirales. IBV and other avian coronaviruses of turkeys and pheasants are classified as ~~group 3~~ *Gammacoronaviruses*, with mammalian coronaviruses comprising ~~groups 1, 2~~ *Alpha* and *Betacoronaviruses*. ~~and 4 (group 4 being the more recently identified severe acute respiratory syndrome (SARS) coronavirus) (Cavanagh, 2003).~~ Novel related coronaviruses have been discovered in wild birds and pigs and have been designated *Deltacoronaviruses* (Woo *et al*., 2012), interestingly the avian *Deltacoronaviruses* have a different genomic order and show no close relationship to the *Gammacoronaviruses*. Coronaviruses have a non-segmented, positive-sense, single-stranded RNA genome.

IB affects chickens of all ages, which, apart from pheasants (Britton & Cavanagh, 2007; Cavanagh *et al*., 2002) are the only species reported to be naturally affected. The disease is transmitted by the air-borne route, direct chicken to-chicken contact and indirectly through mechanical spread (contaminated poultry equipment or egg-packing materials, manure used as fertiliser, farm visits, etc.). IB occurs world-wide and assumes a variety of clinical forms, the principal one being respiratory disease that develops after infection of the respiratory tract tissues following inhalation or ingestion. Infection of the oviduct can lead to permanent damage in immature birds and, in hens, can lead to cessation of egg-laying or production of thin-walled and misshapen shells with loss of shell pigmentation. IB can be nephropathogenic causing acute nephritis, urolithiasis and mortality (Cavanagh & Gelb, 2008 ~~Cavanagh & Naqi, 2003~~). After apparent recovery, chronic nephritis can produce death at a later time. IBV has also been reported to produce disease of the proventriculus (Yu *et al*., 2001). Vaccine and field strains of IBV may persist in the caecal tonsils of the intestinal tract and be excreted in faeces for weeks or longer in clinically normal chickens (Alexander *et al*., 1978). For an in-depth review of IB, refer to Cavanagh & Gelb, 2008 ~~Cavanagh & Naqi, 2003~~. A detailed discussion of IBV antigen, genome and antibody detection assays prepared by De Witt (2000) is also available.

There have been no reports of human infection with IBV.

B. DIAGNOSTIC TECHNIQUES

Confirmation of diagnosis is based on virus isolation, often assisted by serology. Extensive use is made of live and inactivated vaccinations, which may complicate diagnosis by serological methods as antibodies to vaccination and field infections can not always be distinguished. Persistence of live vaccines may also confuse attempts at recovering and/or identifying the causative field strain of IBV.

Table 1. *Infectious bronchitis virus (IBV) test methods available and their purpose*

Method	Purpose					
	Population freedom from infection	Individual animal freedom of infection	Efficiency of eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Virus isolation (embryos or TOCs)	+[a]	++[c]	–	+++	+	+[h]
Staining by immunohistochemistry	–	–	–	++	+	+[h]
Detection of virus genome (RT-PCR)	+[a]	++	++	++	+	+[h]
Virus identification (haemagglutination	–	–	–	–	+	–

Method	Purpose					
	Population freedom from infection	Individual animal freedom of infection	Efficiency of eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
test)						
Virus identification (VN)	–	–	–	–	+	–
Virus identification (gene sequencing)	–	–	–	–	++[f]	–
Antibody detection (AGID)	+[b]	+	–	+[e]	+	–
Antibody detection (VN)	–	–[d]	–	+[e]	-	++[e]
Antibody detection (HIT)	–	-[d]	+	+[e]	+	++[e]
Antibody detection (ELISA)	++[b]	++	++	++[e]	++[g]	++[e]

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose. Although not all of the tests listed as category +++ or ++ have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

[a]Suitable for ensuring lack of infection during the past 10 days; [b]suitable for ensuring lack of infections dating back to more than 10 days; [c]suitable at the individual level only during excretion periods; [d]limited suitability for this purpose as it may be too specific of the serotype used as an antigen; [e]Suitable provided paired samples collected a few weeks apart can be analysed; [f]especially suitable for surveillance of a given or an emerging genotype; [g]especially suitable when IB surveillance is not focused on a given serotype; [h]sometimes used in the evaluation of vaccines to assess protection against viral excretion, but can be positive even when good clinical protection is achieved. TOC = tracheal organ culture; RT-PCR = reverse-transcription polymerase chain reaction; VN = virus neutralisation; AGID = agar gel immunodiffusion; HIT = haemagglutination inhibiton test; ELISA = enzyme-linked immunosorbent assay.

1. Identification of the agent

a) Sampling

Samples appropriate to the form of IB observed must be obtained as soon as signs of clinical disease are evident. Samples must be placed in cold transport media and be frozen as soon as possible. The cold chain from bird to laboratory should be maintained. For acute respiratory disease, swabs from the upper respiratory tract of live birds or tracheal and lung tissues from diseased birds should be harvested, placed in transport medium containing penicillin (10,000 International Units [IU]/ml) and streptomycin (10 mg/ml) and kept on ice and then frozen. For birds with nephritis or egg-production problems, samples from the kidneys or oviduct, respectively, should be collected in addition to respiratory specimens. In some cases, IBV identification by reverse-transcription polymerase chain reaction (RT-PCR) may be desirable without virus isolation. In this case, swabbings from the respiratory tract or cloaca may also be submitted alone, without being placed in liquid transport media (Cavanagh *et al*., 1999). In situations where IB-induced nephritis is suspected, kidney samples should also be selected from fresh carcases for histopathological examination as well as virus isolation. Blood samples from acutely affected birds as well as convalescent chickens should be submitted for serological testing. A high rate of virus recovery has been reported from the caecal tonsil or faeces (Alexander *et al*., 1978). However, isolates from the intestinal tract may have no relevance to the latest infection or clinical disease. IBV isolation may be facilitated using sentinel specific pathogen free (SPF) chickens placed at one or more times in contact with commercial poultry (Gelb *et al*., 1989).

b) Culture

Suspensions of tissues (10–20% w/v) are prepared in sterile phosphate buffered saline (PBS) or nutrient broth for egg inoculation, or in tissue culture medium for chicken tracheal organ culture (TOC) inoculation (Cook *et al*., 1976). The suspensions are clarified by low-speed centrifugation and filtration through bacteriological filters (0.2 µ) before inoculation of SPF embryonated chicken eggs or TOCs.

SPF embryonated chicken eggs and/or TOCs are used for primary isolation of IBV. Cell cultures are not recommended for primary isolation as it is often necessary to adapt IBV isolates to growth in chicken embryos before cytopathic effect (CPE) is produced in chick embryo kidney cells.

Embryonated eggs used for virus isolation should originate preferably from SPF chickens or from breeder sources that have been neither infected nor vaccinated with IBV. Most commonly, 0.1–0.2 ml of sample supernatant is inoculated into the allantoic cavity of 9–11-day-old embryos. Eggs are candled daily for 7 days with mortality within the first 24 hours being considered nonspecific. The initial inoculation usually has limited macroscopic effects on the embryo unless the strain is derived from a vaccine and is already egg adapted. Normally, the allantoic fluids of all eggs are pooled after harvesting 3–6 days after infection; this pool is diluted 1/5 or 1/10 in antibiotic broth and further passaged into another set of eggs for up to a total of three to four passages. Typically, a field strain will induce observable embryonic changes consisting of stunted and curled embryos with feather dystrophy (clubbing) and urate deposits in the mesonephros on the second to fourth passage. Embryo mortality in later passages may occur as the strain becomes more egg adapted. Other viruses, notably adenoviruses that are common to the respiratory tract, also produce embryo lesions indistinguishable from IBV. The IBV-laden allantoic fluid should not agglutinate red blood cells and isolation of IBV must be confirmed by serotyping or genotyping. Infective allantoic fluids are kept at –20°C or below for short-term storage, –60°C for long-term storage or at 4°C after lyophilisation.

TOCs prepared from 19- to 20-day-old embryos can be used to isolate IBV directly from field material (Cook *et al*., 1976). An automatic tissue-chopper is desirable for the large-scale production of suitable transverse sections or rings of the trachea for this technique (Darbyshire *et al*., 1978). The rings are about 0.5–1.0 mm thick, and are maintained in a medium consisting of Eagle's N-2-hydroxyethylpiperazine N'-2-ethanesulphonic acid (HEPES) in roller drums (15 rev/hour) at 37°C. Infection of tracheal organ cultures usually produces ciliostasis within 24–48 hours. Ciliostasis may be produced by other viruses and suspect IBV cases must be confirmed by serotyping or genotyping methods.

c) Methods for identification

The initial tests performed on IBV isolates are directed at eliminating other viruses from diagnostic consideration. Chorioallantoic membranes from infected eggs are collected, homogenised, and tested for avian adenovirus group 1 by immunodiffusion or PCR. Group 1 avian adenovirus infections of commercial chickens are common, and the virus often produces stunted embryos indistinguishable from IBV-infected embryos. Furthermore, harvested allantoic fluids do not hemagglutinate (HA) chick red blood cells. Genetic based tests (RT-PCR or RT-PCR-RFLP [restriction fragment length polymorphism]) are used commonly to identify an isolate as IBV. Other techniques may be used, for example cells present in the allantoic fluid of infected eggs may be tested for IBV antigen using fluorescent antibody tests (Clarke *et al*., 1972) and direct negative-contrast electron microscopy will reveal particles with typical coronavirus morphology in allantoic fluid or TOC fluid concentrates. The presence of IBV in infective allantoic fluid may be detected by RT-PCR amplification and use of a DNA probe in a dot-hybridisation assay (Jackwood *et al*., 1992). Direct immunofluorescence staining of infected TOCs for the rapid detection of the presence of IBV has been described (Bhattacharjee *et al*., 1994). Immuno-histochemistry, with a group-specific monoclonal antibody (MAb), can be used to identify IBV in infected chorioallantoic membranes (Naqi, 1990).

d) Serotype identification

Antigenic variation among IBV strains is common (Cavanagh & Gelb, 2008 ~~Cavanagh & Naqi, 2003~~; Cook, 1984; Dawson & Gough, 1971; Hofstad, 1958; Ignjatovic & Sapats, 2000), but at present there is no agreed definitive classification system. Nevertheless, antigenic relationships and differences among strains are important, as vaccines based on one particular serotype may show little or no protection against viruses of a different antigenic group. As a result of the regular emergence of antigenic variants, the viruses, and hence the disease situation and vaccines used, may be quite different in different geographical locations. Ongoing assessment of the viruses present in the field is necessary to produce vaccines that will be efficacious in the face of antigenic variants that arise. Serotyping of IBV isolates and strains has been done using haemagglutination inhibition (HI) (Alexander *et al*., 1983; King & Hopkins, 1984) and virus neutralisation (VN) tests in chick embryos (Dawson & Gough, 1971), TOCs (Darbyshire *et al*., 1979) and cell cultures (Hopkins, 1974). Neutralisation of fluorescent foci has also been applied to strain differentiation (Csermelyi *et al*., 1988).

MAbs, usually employed in enzyme-linked immunosorbent assays (ELISA), have proven useful in grouping and differentiating strains of IBV (Ignjatovic *et al*., 1991; Koch *et al*., 1986). The limitations of MAb analysis for IB serotype definition are the lack of availability of MAbs or hybridomas and the need to produce new MAbs with appropriate specificity to keep pace with the ever-growing number of emerging IB-variant serotypes (Karaca *et al*., 1992).

e) Genotype identification

RT-PCR genotyping methods have largely replaced HI and VN serotyping for determining the identity of a field strain. The molecular basis of antigenic variation has been investigated, usually by nucleotide sequencing of the gene coding for the spike (S) protein or, more specifically, nucleotide sequencing of the gene coding for the S1 subunit of the S protein (Cavanagh, 1991; Kusters *et al*., 1989) where most of the epitopes to which neutralising antibodies bind are found (Koch *et al*., 1992). An exact correlation with HI or VN results has not been seen, in that while different serotypes generally have large differences (20–50%) in the deduced amino acid sequences of the S1 subunit (Kusters *et al*., 1989), other viruses that are clearly distinguishable in neutralisation tests show only 2–3% differences in amino acid sequences (Cavanagh, 1991). However, there is, in general, good agreement between data represented by the S1 sequence and the VN serotype, and it may eventually be possible to select vaccine strains on the basis of sequence data.

The primary advantages of genotyping methods are a rapid turnaround time, and the ability to detect a variety of genotypes, depending on the tests used. RFLP RT-PCR differentiates IBV serotypes based on unique electrophoresis banding patterns of restriction enzyme-digested fragments of S1 following amplification of the gene by RT-PCR (Jackwood *et al*., 1997; Kwon *et al*., 1993). The RFLP RT-PCR procedure may be used in conjunction with a biotin-labelled DNA probe to first detect IBV in egg fluids harvested following the inoculation of eggs with clinical samples (Jackwood *et al*., 1992). The RFLP RT-PCR test is sometimes used to identify the different serotypes of IBV as well as variant viruses, however nucleotide sequencing is now preferred (see below).

S1 genotype-specific RT PCR may be used to identify specific IBV serotypes (Keeler *et al*., 1998). S1 gene primers specific, for example, for serotypes Massachusetts (Mass), Connecticut, Arkansas, and JMK ~~are~~ may be used in conjunction with a universal primer set that amplifies all IBV serotypes. Primers for the DE/072/92 and California serotypes have also been developed, and other primer sets may be used, based on the contemporary IBV serotypes circulating in a region. Other variant serotypes may be determined to be IBV using the general primers, but the specific serotype cannot be identified. Infections caused by multiple IBV serotypes may be identified.

Nucleotide sequencing of a diagnostically relevant fragment of the S1 gene is the most useful technique for the differentiation of IBV strains and has become the genotyping method of choice in many laboratories. Nucleotide sequencing has also produced evidence that recombination between IB strains occurs often (Cavanagh *et al*., 1992; Zwaagstra *et al*., 1992). RT-PCR product cycle sequencing of the hypervariable amino terminus region of S1 may be used diagnostically to identify previously recognised field isolates and variants (Kingham *et al*., 2000). Comparison and analysis of sequences of unknown field isolates and variants with reference strains for establishing potential relatedness are significant advantages of sequencing.

Recently, it has been shown that coronaviruses isolated from turkeys and pheasants are genetically similar to IBV, having approximately 90% nucleotide identity in the highly conserved region II of the 3' untranslated region (UTR) of the IBV genome (Cavanagh *et al*., 2001; 2002). The potential role of these coronaviruses in IBV infections has not been determined.

The major uses of RT-PCR tests are virus identification and its application in the understanding of epidemiological investigations during IBV outbreaks. The RT-PCR tests, as they now exist however, do not provide information on viral pathogenicity.

- **RT-PCR test procedure**

i) *Extraction of viral RNA*

Any RNA extraction method can be used. There are many protocols available in journals, books and on the web. ~~However, for extracting high quality RNA from allantoic fluid, the Qiagen Viral RNA Mini Kit (www.qiagen.com) is recommended. The Qiagen RNeasy Mini Kit is recommended for extracted IBV RNA from tissue or swabbings.~~ All extracted RNA should be stored between –20°C and –80°C until tested. It is advised that for long-term storage, RNA be kept at –80°C.

ii) *Custom oligos*

Custom oligos can be purchased through any commercial supplier. ~~Operon (www.operon.com) has been making quality custom oligos for years.~~ The target gene for IBV characterisation is the S1 subunit of the spike glycoprotein gene. A commonly used primer pair for amplification of genotypically diverse IBV strains is oligo S15' mod (forward): 5'-TGA-AAA-CTG-AAC-AAA-AGA-3' and CK2 (reverse): 5'-CNG-TRT-TRT-AYT-GRC-A-3' (Gelb *et al*., 2005). The oligo S15'mod/CK2 amplicon is approximately 700 bp in length beginning from the start of the S1 gene spanning two hypervariable regions used for genotyping.

iii) *Reverse-transcription polymerase chain reaction*

Many one and two-step RT-PCR kits are commercially available from manufacturers claiming superior enzyme sensitivity and fidelity. ~~The recommended RT-PCR kit is the basic, two-step, RNA PCR kit from Applied Biosystems (http://www.appliedbiosystems.com).~~ Reverse transcription is performed according to the manufacturer's instructions. RT priming is accomplished with the use of random hexamers (supplied with the kit) or with the reverse PCR primer, in this case CK2 (Keeler *et al*., 1998). One cycle of RT is performed with the following parameters: 25°C for 10 minutes, 42°C for 25 minutes, 95°C for 5 minutes, hold at 4°C. The full RT reaction volume is added to the PCR sample master mix. PCR is performed using the following parameters: 95°C for 2 minutes, 45 cycles of 95°C for 30 seconds, 52°C for 30 seconds, 68°C for 30 seconds, final extension of 68°C for 12 minutes, hold at 4°C. Samples ~~are concentrated in a desiccator overnight or by the use of a vacuum centrifuge. Dried samples~~ are resuspended in ~~12 µl of DEPC treated water and~~ 6 µl of loading buffer prior to electrophoresis on a 1.8% agarose gel containing ~~ethidium bromide~~ nucleic acid stain. Gels are visualised with a UV light box. Bands are compared to a commercially available 100 bp ladder and an IBV positive control.

iv) *S1 gene sequencing*

Bands visualised in the agarose gel that are of similar size to the positive control are excised from the gel. The PCR product is ~~separated~~ isolated from the agarose gel using ~~the Qiagen Gel Extraction kit (www.qiagen.com) or any other~~ a commercial gel extraction kit. Purified PCR products are run on a second 1.8% agarose ~~ethidium bromide~~ nucleic acid stain gel to determine the quantity of product present. Approximately 20 µl (10 ng/µl) of PCR product is required for sequencing. Sequencing can be performed at ~~the University of Delaware Sequencing & Genotyping Center (http://www.udel.edu/dnasequence) or another~~ a university or commercial sequencing facility. Sequence chromatograms are edited using ~~the DNAStar~~ suitable analysis software. ~~or on-line freeware 4peaks (http://www.mekentosj.com/4peaks/) or chromas lite (http://www.technelysium.com.au/chromas_lite.html).~~ Edited sequences of IBV isolates are characterised using BLASTn for nucleotide or BLASTp for protein analysis (http://www.ncbi.nlm.nih.gov/BLAST/).

2. Serological tests

A number of tests have been described. Those considered here include VN (Dawson & Gough, 1971), agar gel immunodiffusion (AGID) (Witter, 1962), HI (Alexander *et al*., 1983) and ELISA (Mockett & Darbyshire, 1981). Each test has advantages and disadvantages in terms of practicality, specificity, sensitivity and cost. In general, for routine serological testing, the VN tests are too expensive and impractical, and AGID tests lack sensitivity. ELISA and HI tests are most suitable for routine serology. ELISAs are useful for general monitoring of IBV exposure and can detect antibody responses to all serotypes. HI when used on serial sera from young growing chickens such as pullets and broilers can give information on the serotype-specific antibody status of a flock. Regular monitoring of sera from flocks for IB antibody titres may help to indicate the level of vaccine or field challenge responses. Because chicken sera from older birds contain antibodies that are highly cross-reactive against antigenically unrelated strains, serodiagnosis of suspected disease outbreaks of IB cannot be used with a high degree of confidence.

a) Virus neutralisation

In VN tests, all sera should first be heated to 56°C for 30 minutes. Virus is mixed with serum and incubated for 30–60 minutes at 37°C or room temperature. Chicken embryos are most often employed, but antibodies can be measured using TOC or cell culture systems. Two methods have been used to estimate neutralising antibodies. One employs a constant serum concentration reacted with varying dilutions of virus (the alpha method) and the other employs a constant amount of virus and varying dilutions of serum (the beta method).

In the alpha method, tenfold dilutions of egg-adapted virus are reacted with a fixed dilution (usually 1/5) of antiserum, and the mixtures are inoculated into groups of from five to ten eggs. The virus alone is titrated in parallel. End-points are calculated by the Kärber or the Reed and Muench methods. The results are expressed as a neutralisation index (NI) that represents the $\log_{10}$ difference in the titres of the virus alone and that of the virus/antiserum mixtures. The NI values may reach 4.5–7.0 in the case of homologous virus/serum mixtures; values of <1.5 are not specific, but a heterologous virus will give a value as low as 1.5.

The beta method is the more widely used neutralisation test for antibody assay with chicken embryos. Two- or four-fold dilutions of antiserum are reacted in equal volumes with a dilution of virus, usually fixed at 100 or 200 EID_{50} (median embryo-infective doses) per 0.05 ml and 0.1 ml of each mixture inoculated into the allantoic cavity of each of from five to ten embryonated eggs. A control titration of the virus is performed simultaneously to confirm that the fixed virus dilution in the virus/serum mixtures was between $10^{1.5}$ and $10^{2.5}$ EID_{50}. End-points of the serum titres are determined by the Kärber or Reed and Muench method as before, but here are expressed as reciprocals of $\log_2$ dilutions. This fixed-virus/varying-serum method is also employed for neutralisation tests in tracheal organ cultures using five tubes per serum dilution, as is conventional with other viruses (Darbyshire *et al*., 1979). The results are calculated according to Reed and

Muench, and the virus titres are expressed as median ciliostatic doses per unit volume (log_{10} CD_{50}). Serum titres are again expressed as log_2 dilution reciprocals. This test is more sensitive than others, but technical logistics hamper its more widespread adoption.

b) Haemagglutination inhibition

A standard protocol for a HI test for IBV has been described (Alexander *et al*., 1983), and the test procedure detailed below is based on that standard. Strains and isolates of IBV will agglutinate chicken red blood cells (RBCs) after neuraminidase treatment (Ruano *et al*., 2000; Schultze *et al*., 1992). The strain selected to produce antigen may be varied, depending on the requirements of diagnosis. The antigen for the HI test is prepared from IBV-laden allantoic fluids.

For HA and HI tests, procedures are carried out at 4°C.

- **Haemagglutination test**

i) Dispense 0.025 ml of PBS, pH 7.0–7.4, into each well of a plastic U or V-bottom microtitre plate.

ii) Place 0.025 ml of virus antigen in the first well. For accurate determination of the HA content, this should be done from a close range of an initial series of dilutions, i.e. 1/3, 1/4, 1/5, 1/6, etc.

iii) Make twofold dilutions of 0.025 ml volumes of the virus antigen across the plate.

iv) Dispense a further 0.025 ml of PBS into each well.

v) Dispense 0.025 ml of 1% (v/v) chicken RBCs to each well.

vi) Mix by tapping the plate gently and allow the RBCs to settle for 40–60 minutes at 4°C, when control RBCs should be settled to a distinct button.

vii) HA is more easily determined by tilting the plate and observing the presence or absence of tear-shaped streaming of the RBCs. The titration should be read to the highest dilution giving complete HA in which there is no sedimentation or streaming; this is 100% HA and represents 1 HA unit (HAU) and can be calculated accurately from the initial range of dilutions.

- **Haemagglutination-inhibition test**

The HI test is used in the diagnosis and routine flock monitoring of vaccine responses.

i) Dispense 0.025 ml of PBS into each well of a plastic U or V-bottom microtitre plate.

ii) Place 0.025 ml of serum into the first well of the plate.

iii) Make twofold dilutions of 0.025 ml volumes of the serum across the plate.

iv) Add 4 HAU of virus antigen in 0.025 ml to each well and leave for 30 minutes.

v) Add 0.025 ml of 1% (v/v) chicken RBCs to each well and, after gentle mixing, allow the RBCs to settle for 40–60 minutes when control RBCs should be settled to a distinct button.

vi) The HI titre is the highest dilution of serum causing complete inhibition of 4 HAU of antigen. The agglutination is assessed more exactly by tilting the plates. Only those wells in which the RBCs 'stream' at the same rate as the control wells (containing 0.025 ml RBC and 0.05 ml PBS only) should be considered to show inhibition.

vii) The validity of results should be assessed against a negative control serum, which should not give a titre $>2^2$, and a positive control serum, for which the titre should be within one dilution of the known titre.

viii) Sera are usually regarded as positive if they have a titre of 2^4 or more. However, it should be noted that even in SPF flocks, a very small proportion of birds may show a nonspecific titre of 2^4, but usually in birds over 1 year of age.

c) Enzyme-linked immunosorbent assay

The ELISA technique is a sensitive serological method and gives earlier reactions and higher antibody titres than other tests (Mockett & Darbyshire, 1981). It lacks type or strain specificity, but is valuable for monitoring vaccination responses under field conditions. Commercial kits for ELISAs are available – these are based on several different strategies for the detection of IBV antibodies. Usually, such tests have been evaluated and validated by the manufacturer, and it is therefore important that the instructions specified for their use be followed carefully. The ELISA is widely used to identify IBV-infected flocks (broilers) based on high antibody titres. If IB reoccurs in the next flock on the farm, virus isolation attempts are performed and the virus is genotyped by RFLP or S1 sequencing.

d) Agar gel immunodiffusion

AGID can be used in diagnosis (Witter, 1962). The antigen is prepared from a homogenate of the chorioallantoic membranes of infected chicken embryos. The Beaudette embryo-lethal strain is often employed to produce antigen. The test lacks sensitivity and is liable to yield inconsistent results as the presence and duration of precipitating antibodies may vary with individual birds.

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

1. Background

All live and inactivated commercial vaccines must be licensed. Strains used in live virus vaccines generally require attenuation. At present, many countries only permit live vaccines of the Massachusetts type, such as the H120. Some countries may also have licensed vaccines to other live strains such as Connecticut, Arkansas, or Delaware 072 (USA) or the 4/91 strain (United Kingdom). Live vaccines may be given as aerosols, in the drinking water, or by the intraocular route (eyedrop).

The efficacy of inactivated vaccines depends heavily on proper priming with a live vaccine(s). Inactivated vaccines must be administered to birds individually, by intramuscular or subcutaneous injection. Variant strains may be used to prepare inactivated autogenous vaccines for controlling IB in layers and breeders, subject to local legislative requirements.

Live vaccines confer better local immunity in the respiratory tract and also may protect against a wider antigenic spectrum of field strains (Cook *et al*., 1999). However, vaccination with live vaccines may not protect layer flocks against variant serotype challenge especially common on farms with flocks of multiple ages where production drops as early as 40 weeks of age are not uncommon (Gelb *et al*., 1991). Live vaccines carry a risk of residual pathogenicity associated with vaccine back-passage in flocks. However, proper mass application techniques (e.g. spray or drinking water) can achieve uniform distribution of the vaccine in the flock and avoid back-passage. Furthermore, the use of vaccines at manufacturer's recommended dosages will also help avoid back-passage reversion that may be caused by fractional dose application.

There are prospects for genetically engineered vaccines (Armesto *et al*., 2011; Casais *et al*., 2003), and *in-ovo* vaccination (Tarpey *et al*., 2006; Wakenell *et al*., 1995).

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.6 are intended to be general in nature. National and international standards that apply in the country in which IB vaccines are manufactured must be complied with. The licensing authority should provide information and guidance on requirements. These are now often presented in general terms, as applying to all vaccines – avian and mammalian, live and inactivated, or viral and bacterial vaccines. There may also be specific requirements applying to IB vaccines, live and inactivated. As examples, references are given to the European and USA regulations (Commission of the European Communities ~~1999~~ [Interim Publication Nov. 1993]; ~~Council of Europe~~ European Pharmacopoeia [2010a; 2010b]; USDA Code of Federal Regulations 113.327).

The list of extraneous agents that must be shown to be absent continues to grow. Manufacturers must be familiar with those that currently apply in their country. Recent additions are avian nephritis virus and avian pneumovirus.

For IB vaccines, important differences among countries may arise regarding the challenge virus to be used for potency tests, and its validation. Traditionally, the virulent M41 (Mass 41) strain of the Massachusetts type has been used for challenge tests of both live and inactivated vaccines. Although this type is still common, it is often not the only or the dominant type in many countries and it may be advisable to prepare vaccines from other types. It is logical for challenges to be made by the same type as present in the vaccine. Establishing criteria for validating the challenge virus may be more difficult for non-Massachusetts types, because of their lower virulence in general. Inactivated vaccines are usually expected to protect against drops in egg production. The traditional M-41 challenge ~~as described in this chapter~~ should cause a drop of at least 67% in the unvaccinated controls, which was considered by some IB specialists as being excessive as too dependent on the chicken genetic line and on particular challenge parameters. ~~but~~ When using other types much lower drops in egg production may be regarded as satisfactory, depending on published evidence of the effects of these strains in the field. It therefore seems necessary ~~There is also a tendency~~ to relax the criteria for Massachusetts type challenges, and the European Pharmacopoeia now defines a satisfactory drop in egg production for Massachusetts types to be at least 35%, and for non-Massachusetts types to be at least 15%, provided that the drop is 'commensurate with the documented evidence' (European Pharmacopoeia).

2. Outline of production and minimum requirements for vaccines

a) Characteristics of the seed

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 *Principles of veterinary vaccine production* and Chapter 1.1.7 *Tests for sterility and freedom from contamination of biological materials*.

The seed-lot (master seed) system should be employed for whatever type of vaccine is produced. Each virus must be designated as to strain and origin and must be free from contamination with other strains of IBV and extraneous agents. Separate storage facilities should be provided between the strains of virus intended for vaccines or for challenge.

For live virus vaccines, many countries permit only strains of the Massachusetts type. Some countries allow other strains, usually on the basis that those strains are already present in their national flocks. The antigenic type incorporated in both live and inactivated vaccines requires justification if there is doubt as to its existence in a country.

~~**b) Method of culture**~~

~~All seed viruses are grown in the allantoic sac of developing chicken embryos or in suitable cell cultures. The eggs should be from an SPF flock.~~

~~**c) Validation as a vaccine**~~

~~**o Purity**~~

i) *Biological characteristics of the master seed*

Live vaccines: Currently live IBV vaccines are normally attenuated by multiple repeat passage of a virulent virus in specific pathogen free (SPF) embryonated chicken eggs (Cavanagh, 2003). Spontaneous mutations arise throughout the IBV genome some of which lead to attenuation of the virus. However, as a consequence of this method the attenuated viruses produced by this approach have only a few mutations that are responsible for loss of virulence and these will differ between vaccine strains. Two major drawbacks of this method is that once the virus is used to inoculate chickens the mutations within the attenuated vaccine viruses may back-mutate resulting in virulent virus, an undesirable consequence, or that as a consequence of multiple passage the immunogenicity of the attenuated virus will not result in adequate protection. Given the nature of these live attenuated vaccines, further passage beyond the master seed stock must be kept to a minimum to prevent potential loss of immunogenicity. The stocks must be grown in SPF chicken eggs to prevent the introduction of other potential pathogens.

Inactivated vaccines: IBV inactivated vaccines are also used to combat IBV usually in layer hens and used as a boost vaccination or to induce some protection against other IBV strains. Inactivated IBV vaccines have poor efficacy unless the chickens have previously been primed by vaccination with a live virus vaccine. The IBVs, for inactivated vaccines, are grown in non-SPF eggs and are chemically inactivated usually by destroying the genomic RNA. Batches of inactivated vaccines must be tested for residual infectivity using embryonated eggs.

Every seed lot must be free from bacterial, fungal, mycoplasmal and viral contamination.

For the detection of extraneous viruses, the seed is first treated with a high-titred monospecific antiserum prepared against the strain under examination or against one of identical type. This mixture is cultured in a variety of ways, designed to confirm the absence of any viruses considered from past experience to be potential contaminants. The antiserum must not contain antibodies to adenovirus, avian encephalomyelitis virus, avian rotavirus, chicken anaemia virus, fowlpox virus, infectious laryngotracheitis virus, influenza A virus, Newcastle disease virus, infectious bursal disease virus, leukosis virus, reovirus, Marek's disease virus, turkey herpesvirus, adeno-associated virus, egg-drop syndrome 76 (EDS76) virus, avian nephritis virus, avian pneumovirus or reticulo-endotheliosis virus. The inoculum given to each unit of the culture system used should contain a quantity of the neutralised IBV component under test that had an initial infectivity of at least ten times the minimum field dose. These systems include:

1. SPF chicken embryos, incubated for 9–11 days, inoculated via both allantoic sac and chorioallantoic membrane (two passages);
2. Chicken embryo fibroblast cultures, for leukosis virus subgroups A and B. The COFAL test (test for avian leukosis using complement fixation) or double-antibody sandwich ELISA for group-specific leukosis antigen is performed on cell extracts harvested at 14 days. An immunofluorescence test for reticulo-endotheliosis virus is done on cover-slip cultures after two passages.
3. SPF chicken kidney cultures that are examined for CPEs, cell inclusions and haemadsorbing agents passaged at intervals of no fewer than 5 days for up to 20 days' total incubation.

4. SPF chickens of minimum vaccination age inoculated intramuscularly with 100 field doses, and on to the conjunctiva with ten field doses; this is repeated 3 weeks later when the chickens are also inoculated both into the foot pad and intranasally with ten field doses. Observations are made for 6 weeks overall, and serum is collected for tests for avian encephalomyelitis, infectious bursal disease, Marek's disease, Newcastle disease and *Salmonella pullorum* infection.

Vaccines intended to protect against loss of egg production should be tested for duration of antibody response. Mean HI titres should be >6 $\log_2$ up to at least 60 weeks of age. Serological tests should be done at intervals frequent enough to show that titres have not been boosted by extraneous IBV infection.

Vaccines intended for protection of broiler chickens or rearing chickens against the respiratory form of the disease should be similarly tested for duration of antibody responses; in the case of broilers this would be up to the normal age for slaughtering, and in the case of pullets up to the age when a booster vaccination would be administered (often at 16–18 weeks of age).

~~o **Safety**~~

ii) *Validation as a vaccine strain*

Tests on seed virus should include a test for any potential ability to revert to virulence. Live and inactivated vaccine seed must be tested for safety as in Section C.2.b.iv.

~~o **Efficacy**~~

To demonstrate efficacy, a trial vaccine must be made from the master seed and the working seed at five passages from the master seed and subjected to tests that demonstrate their protective effect.

For live vaccines, a minimum of ten SPF chickens aged 3–4 weeks are vaccinated intranasally or by eyedrop with the recommended dose. Ten unvaccinated control birds from the same age and source are retained separately. All birds of both groups are challenge inoculated either intranasally or by eyedrop 3–4 weeks later, with $10^{3.0}$–$10^{3.5}$ EID_{50} ~~virulent Massachusetts M-41~~ of reference challenge virus, representative of the vaccine virus antigenic group. A swab of the trachea is taken from each bird 4–5 days after challenge and placed in 3 ml of antibiotic broth. Each fluid is tested for IBV by the inoculation (0.2 ml) of five embryonated eggs, 9–11 days of age. An alternative test to that of taking swabs is to kill birds at 4–6 days after challenge and examine microscopically the tracheal rings for ciliary activity (Darbyshire, 1985). Failure to resist challenge is indicated by an extensive loss of ciliary motility. The live vaccine is suitable for use if at least 90% of the challenge vaccinated birds show no evidence of IBV in their trachea, while 90% or more of the control birds should have evidence of the presence of the virus.

To assess an inactivated vaccine intended to protect laying birds, 30 or more SPF chickens are vaccinated as recommended at the earliest permitted age. If a primary vaccination with live vaccine is first undertaken, an additional group of birds is given only the primary vaccination. In both cases, these primary vaccinations should be done at no later than 3 weeks of age. The inactivated vaccine is given 4–6 weeks after the live priming vaccination. A further group of 30 control birds are left unvaccinated. All groups are housed separately until 4 weeks before peak egg production, and then are housed together. Individual egg production is monitored and once it is regular, all birds are challenged, egg production being recorded for a further 4 weeks. The challenge should be sufficient to ensure loss of production during the 3 weeks after challenge. The loss in the control group should be at least ~~67%;~~ 35% where challenge has been made with a Massachusetts-type strain. Where it is necessary to carry out a challenge with a strain of another serotype for which there is documented evidence that the strain will not cause a 35% drop in egg production, the challenge must produce a drop in egg production commensurate with the documented evidence and in any case not less than 15%; the group that received primary live virus vaccine followed by inactivated vaccine should remain at the previous level, and the group given only a primary vaccination should show an intermediate drop in production. The vaccine complies with the test if egg production or quality is significantly better in the group having received the inactivated vaccine than in any control group. Sera are collected from all birds at vaccination, 4 weeks later, and at challenge; there should be no response in the control birds.

To assess an inactivated vaccine intended to protect birds against respiratory disease, 20 SPF chickens aged 4 weeks are vaccinated as recommended. An additional 20 control birds of the same age and origin are housed with this first group. Antibody responses are determined 4 weeks later; there should be no response in the control birds. All birds are then challenged with 10^3 CID_{50} (50% chick infective dose) of virulent virus, killed 4–7 days later, and tracheal sections are examined for ciliary motility. At least 80% of the unvaccinated controls should display complete ciliostasis, whereas the tracheal cilia of a similar percentage of the vaccinated birds should remain unaffected.

Both live and inactivated vaccines containing Newcastle disease, infectious bursal disease, reovirus and EDS76 viruses are available in some countries. The efficacy of the different components of these vaccines must each be established independently and then as a combination in case interference between different antigens exists.

~~2.~~b) Method of manufacture

i) *Procedure*

All virus strains destined for live vaccines are cultured in the allantoic sac of SPF chicken embryos or in suitable cell cultures. For inactivated vaccines, hens' eggs from healthy non-SPF flocks may be used. The pooled fluid is clarified and then titrated for infectivity. For live vaccines this fluid is lyophilised in vials, and for inactivated vaccines it is blended with high-grade mineral oil to form an emulsion to which a preservative is added.

ii) *Requirements for ingredients*

See chapter 1.1.6 with special focus on products of biological origin (POBs) originating from a country with negligible risk for transmissible spongiform encephalopathies (TSEs).

iii) *In-process controls*

The required antigen content is based on initial test batches of vaccine of proven efficacy in laboratory and field trials. Infectivity titrations are done in chicken embryos.

Live vaccine should contain not less than $10^{3.5}$ EID_{50} per dose per bird until the expiry date indicated, and not less than $10^{2.5}$ EID_{50} per dose per bird after incubation at 37°C for 7 days at the time of issue. For inactivated vaccine, the inactivating agent and inactivation procedure must be shown under manufacture to be effective on both IBV and potential contaminants. With the use of beta-propiolactone or formalin, any live leukosis viruses and *Salmonella* species must be eliminated; and with other inactivating agents, the complete range of potential contaminants must be rendered ineffective. Before inactivation procedures, it is important to ensure homogeneity of suspensions, and a test of inactivation should be conducted on each batch of both bulk harvest after inactivation and the final product.

Tests of inactivation should be appropriate to the vaccine concerned and should consist of two passages in cell cultures, embryos or chickens, using inoculations of 0.2 ml and ten replicates per passage.

~~**4. Batch control**~~

iv) *Final product batch tests*

Sterility

Every batch of live vaccine should be tested for the absence of extraneous agents as for the seed virus (see chapter 1.1.7).

Safety

For live attenuated vaccines

Use no fewer than ten chickens from an SPF flock that are of the minimum age stated on the label for vaccination. Administer by eyedrop to each chicken ten doses of the vaccine reconstituted so as to obtain a concentration suitable for the test. Observe the chickens for 21 days. For vaccines intended for chickens that are 2 weeks old or more, use the chickens inoculated in the 'test for extraneous agents using chickens' (see Section C.2.a.i.4). If during the period of observation, more than two chickens die from causes not attributable to the vaccine, repeat the test. The vaccine complies with the test if no chicken shows serious clinical signs, in particular respiratory signs, and no chicken dies from causes attributable to the vaccine.

For inactivated vaccines

Inject a double dose of vaccine by the recommended route into each of ten 14–28-day-old chickens from an SPF flock. Observe the chickens for 21 days. Ascertain that no abnormal local or systemic reaction occurs.

Batch potency

The potency test is developed from the results of efficacy tests on the master seed virus. Live vaccines are tested for potency by titration of infectivity, and inactivated vaccines by measuring antibody production. The potency test for a batch of inactivated vaccine consists of vaccinating 20 SPF chickens, 4 weeks of age, and showing that their mean HI titre 4 weeks later is not less than 6 $\log_2$.

~~**d) Duration of immunity**~~

Vaccine must be shown to have the required potency to achieve the claimed duration of immunity at the end of the claimed shelf life.

~~**e) Stability**~~

At least three batches should be tested for stability and must give satisfactory results for 3 months beyond the claimed shelf life. The stability of a live vaccine must be measured by maintenance of an adequate infectivity titre. The stability of an inactivated vaccine is measured at intervals by batch potency tests. The concentration of preservative and persistence through the shelf life should be assessed. There should be no physical change in the vaccine and it should regain its former emulsion state after one quick shake.

~~**f) Preservatives**~~

There are maximum level requirements for the use of antibiotics, preservatives and residual inactivating agents.

c) Requirements for authorisation/registration/licensing

i) *Manufacturing process*

For registration of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Section C.2.a and b) should be submitted to the authorities. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

ii) *Safety requirements*

A safety test must be carried out on each batch of final product, as in Section C.2.b.iv. A potency test must be carried out on each batch of final product, as in Section C.2.b.iv, at manufacture and at the end of the stated shelf life.

a) Target and non-target animal safety (depending of the different status of the animals: young, pregnant animals, etc.)

b) Reversion-to-virulence for attenuated/live vaccines and environmental considerations (dissemination and spread of live vaccines and their potential to cause problems for non-vaccinated animals)

c) Precautions (hazards)

IBV itself is not known to present any danger to staff employed in vaccine manufacture or testing. Extraneous agents may be harmful, however, and the initial stages of handling a new seed virus should be carried out in a safety cabinet. It is a wise precaution with all vaccine production to take steps to minimise exposure of staff to aerosols of foreign proteins. Persons allergic to egg materials must never be employed in this work. All Vaccines should be identified as harmless or pathogenic for vaccinators. Manufacturers should provide adequate warnings that medical advice should be sought in the case of self-injection (including for adjuvants, oil-emulsion vaccine, preservatives, etc.) with warnings included on the product label/leaflet so that the vaccinator is aware of any danger.

~~**5. Tests on the final product**~~

~~**a) Safety**~~

~~A safety test must be carried out on each batch of final product~~

iii) *Efficacy requirements*

To register a commercial vaccine, a batch or batches produced according to the standard method and containing the minimum amount of antigen or potency value shall prove its efficacy (protection); each future commercial batch shall be tested before release to ensure it has the same potency value demonstrated by the batch(es) used for the efficacy test(s).

Usually vaccine efficacy (protection) is estimated in vaccinated animals directly by evaluating their resistance to live pathogen challenge.

In the case of pathogens with several serotypes, vaccine efficacy should be established for each serotype.

The challenge models for determining efficacy are as out lined in Section C.2.b.ii.

~~**b) Potency**~~

~~A potency test must be carried out on each batch of final product, as in Section C.4.c, at manufacture and at the end of the stated shelf life.~~

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CHAPTER 2.4.15.

MALIGNANT CATARRHAL FEVER

SUMMARY

Definition of the disease: *Malignant catarrhal fever* (*MCF*) *is an acute, generalised and usually fatal disease affecting many species of* Artiodactyla*. The disease has been most often described as affecting species of the subfamily* Bovinae *and family* Cervidae*, but is also recognised in domestic pigs as well as giraffe and species of antelope belonging to the subfamily* Tragelaphinae*. MCF is defined by ~~the recognition of~~ characteristic lymphoid cell accumulations in nonlymphoid organs, vasculitis and T-lymphocyte hyperplasia in lymphoid organs. The disease occurs following infection with certain herpesviruses of the genus* Macavirus*. The best characterised of these are alcelaphine herpesvirus-1 (AlHV-1) and ovine herpesvirus-2 (OvHV-2). ~~the main cause of which is either of two gammaherpesviruses. The alcelaphine herpesvirus-1~~ (AlHV-1, which is maintained by inapparently infected wildebeest, causes the disease in cattle in regions of Africa and in a variety of ruminant species in zoological collections world-wide. OvHV-2, which is prevalent in ~~all varieties of~~ domestic sheep as a subclinical infection, is the cause of MCF in most regions of the world. ~~This form of the disease is also known as sheep-associated MCF.~~ In both forms of the disease, animals with clinical disease are not a source of infection as virus is only excreted by the natural hosts, wildebeest and sheep, respectively.*

Description of the disease: *MCF usually appears sporadically and affects few animals, though both AlHV-1 and OvHV-2 can give rise to epizootics. There is a marked gradation in susceptibility to the OvHV-2 form of MCF ranging from the relatively resistant* Bos taurus *and* B. indicus*, through water buffalo, North American bison and many species of deer, to the extremely susceptible Père David's deer, and Bali cattle. The disease may present a wide spectrum of clinical manifestations ranging from the acute form, when minimal changes are observed prior to death, to the more florid cases characterised by high fever, bilateral corneal opacity, profuse catarrhal discharges from the eye and nares, necrosis of the muzzle and erosion of the buccal epithelium. ~~Infectivity from animals with the AlHVAlHV-1 form of MCF can be recovered only by employing techniques that retain the viability of host cells, while OvHV-2 has never been recovered from affected animals.~~ Diagnosis is normally achieved by observing the characteristic histopathological changes, though detection of viral DNA in either form of the disease has become the preferred option.*

Identification of the agent: *AlHV-1 may be recovered from clinically affected animals using peripheral blood leukocytes or lymphoid cell suspensions ~~prepared from lymph nodes and spleen~~, but cell viability must be preserved during processing, as infectivity cannot be recovered from dead cells. Virus can also be recovered from wildebeest, either from peripheral blood leukocytes or from cell suspensions of other organs. Most monolayer cultures of ruminant origin are probably susceptible and develop cytopathic effect* (*CPE*)*~~, although bovine thyroid cell cultures have been used extensively for recovery of virus~~. Primary isolates typically produce multinucleated CPE in which viral antigen can be identified by immunofluorescence or immunocytochemistry using suitable antisera or monoclonal antibodies. The OvHV-2 agent has never been ~~identified formally~~ isolated in culture, although lymphoblastoid cell lines propagated from affected animals contain OvHV-2-specific DNA. Both agents have been transmitted experimentally to rabbits and hamsters, which develop lesions characteristic of MCF.*

Viral DNA has been detected in clinical material from cases of MCF caused by both AlHV-1 and OvHV-2 using the polymerase chain reaction, and this is becoming the method of choice for diagnosing ~~the OvHV-2~~ both forms of the disease.

Serological tests: *Infected wildebeest, the natural host, consistently develop antibody to AlHV-1, which can be detected in a variety of assays including virus neutralisation, immunoblotting, enzyme-linked immunosorbent assay* (*ELISA*) *and immunofluorescence. Antibody to OvHV-2 can*

be detected by using AlHV-1 as the source of antigen. Domestic sheep consistently have antibody that can be detected by immunofluorescence, ELISA or immunoblotting. However, the antibody response of clinically affected animals is limited, with no neutralising antibody developing, so that detection relies on the use of immunofluorescence, ELISA or immunoblotting ~~Antibody to OvHV-2 has only been detected by using AlHV-1 as the source of antigen. Domestic sheep consistently have antibody that can be detected by immunofluorescence, ELISA or immunoblotting. While antibody often can be detected by immunofluorescence and ELISA in cattle with MCF, in,~~ although antibody is not always present in more acutely affected animals such as deer.~~antibody is not always present. The competitive inhibition enzyme-linked immunosorbent assay (CI-ELISA) appears to have a sensitivity and specificity that are equal to or better than the other tests, although a recently described ELISA gave good concordance with this test.~~

Requirements for vaccines ~~and diagnostic biologicals~~: *No vaccine is currently available for this disease.*

A. INTRODUCTION

Definition of the disease: Malignant catarrhal fever (MCF) is a generally fatal disease of cattle and many other species of *Artiodactyla*, which occurs following infection with certain herpesviruses of the genus *Macavirus*. Six herpesviruses have been recognised as causing MCF, the best characterised being~~either~~ alcelaphine herpesvirus-1 (AlHV-1) and ~~or~~ ovine herpesvirus-2 (OvHV-2). MCF is characterised by systemic lymphoproliferation and is usually fatal, with infected cells being detectable in blood and most tissues at necropsy by polymerase chain reaction (PCR) with virus-specific primers. Natural hosts of these viruses, including Wildebeest (*Connochaetes* spp. of the subfamily *Alcelaphinae*) for AlHV~~the natural hosts of AlHV~~-1 and domestic sheep for OvHV-2, experience no clinical disease following infection~~Likewise, infection of domestic sheep, the natural host of OvHV-2, has not been associated with any clinical reaction following natural infection~~, although experimentally, large doses of OvHV-2 ~~virus~~ produced clinical signs of MCF when inoculated into naive ~~susceptible~~ sheep (Li *et al*., 2005b).

Causal pathogen: Disease caused by AlHV-1 is restricted to those areas of Africa where wildebeest are present and to zoological collections elsewhere, and has been referred to as wildebeest-associated (WA-) MCF. The OvHV-2 form of the disease occurs world-wide wherever sheep husbandry is practised and has been described as sheep-associated (SA-) MCF. Both forms of the disease may present a wide spectrum of clinical entities, though the characteristic histopathological changes are very similar in all cases. These two viruses belong to a ~~sub~~group of closely related ruminant maca~~rhadino~~viruses that infect three subfamilies of *Bovidae* (*Alcelaphinae*, *Hippotraginae* and *Caprinae*); all probably have a potential to cause typical MCF. On rare occasions members of this group of viruses other than AlHV-1 and OvHV-2 have been identified as a cause of MCF.

~~• Clinical and pathological changes~~

Description of the disease: The clinical signs of MCF are highly variable and range from peracute to chronic with, in general, the most obvious manifestations developing in the more protracted cases. In the peracute form, either no clinical signs are detected, or depression followed by diarrhoea and dysentery may develop for 12–24 hours prior to death. In general, the onset of signs is associated with the development of a high fever, increased serous lachrymation and nasal exudate, which progresses to profuse mucopurulent discharges. Animals may be inappetent and milk yields may drop. Characteristically, progressive bilateral corneal opacity develops, starting at the periphery. In some cases skin lesions appear (characterised by ulceration and exudation), which may form hardened scabs associated with necrosis of the epidermis, and are often restricted to the perineum, udder and teats. Salivation associated with hyperaemia may be an early sign, progressing to erosions of the tongue, hard palate, gums and, characteristically, the tips of the buccal papillae. Superficial lymph nodes may be enlarged and limb joints may be swollen.

Nervous signs such as hyperaesthesia, incoordination, nystagmus and head pressing may be present in the absence of other clinical signs or as part of a broader more characteristic syndrome. In addition, a number of cases of MCF with dermatological presentation have recently been described in Sika deer infected with caprine herpesvirus 2 (CpHV-2; Foyle *et al*., 2009 and references cited therein). These cases exhibited cutaneous lesions combined with lymphocytic vasculitis characteristic of MCF, with CpHV-2 being detected by PCR and DNA sequencing.

Wildebeest-associated MCF occurs in the cattle-raising regions of eastern Africa where pastoralists use areas grazed by wildebeest, and in southern Africa in areas where wildebeest and cattle are grazed together. The disease, however, can also affect a variety of other ruminant species in zoological collections world-wide and so,

apart from antelope of the subfamilies Alcelaphinae and Hippotraginae, it is advisable to regard all ruminants as susceptible.

Sheep-associated MCF occurs world-wide in cattle and other species, normally appearing sporadically and affecting only one or a few animals. However, on occasion, incidents occur in which several animals become affected, and this appears to be associated with certain sheep flocks that may continue to transmit disease for a number of years. The disease can also infect and cause substantial losses in North American Bison (*Bison bison*), red deer (*Cervus elaphus*), other deer species and water buffalo (*Bubalus bubalis*) and even more readily in Père David's deer (*Elaphurus davidianus*) and Bali cattle (*Bos javanicus*). OvHV-2 is also responsible for causing MCF in zoological collections, where disease has been reported in a variety of species including giraffe.

~~There is a wide spectrum of susceptibility to OvHV-2 induced disease, ranging from *Bos taurus* and *B. indicus*, which are relatively resistant, through most species of deer, bison (*Bison bison*) and water buffalo (*Bubalus bubalis*), which are much more susceptible, to the extremely susceptible Bali cattle (*Bos javanicus*) and Père David's deer (*Elaphurus davidianus*). The more resistant species tend to experience a more protracted infection and florid lesions, while in the more susceptible species the disease course tends to be shorter and the clinical signs less dramatic.~~

Reports from several countries, and in particular from Norway, that the disease affects domestic pigs have recently been confirmed by the detection of virus DNA in affected animals (Loken *et al*., 1998; Syrjala *et al*., 2006). Experimental infection of pigs with OvHV-2 has also been documented (Li *et al.*, 2012). Signs are very similar to those seen in acutely affected cattle.

The more resistant species tend to experience a more protracted infection and florid lesions, while in the more susceptible species the disease course tends to be shorter and the clinical signs less dramatic. A mild form of the disease described in 1930 was regarded with some scepticism because the disease could be confirmed only by histological changes observed at post-mortem. However, recent investigations using molecular and serological methods would appear to confirm that a few infected animals may recover following mild or even quite severe clinical reactions (Michel *et al*., 1994). Some studies indicate that substantial numbers of animals may become infected without developing clinical disease.

~~• **Pathology**~~

Gross pathological changes reflect the severity of clinical signs, but are generally widespread and may involve most organ systems. Erosions and haemorrhages may be present throughout the gastrointestinal tract, and in the more acute cases can be associated with haemorrhagic intestinal contents. In general, lymph nodes are enlarged, although the extent of lymph node involvement varies within an animal. ~~Lymph nodes can often be firm and white when cross sectioned, while others, in particular submandibular and retropharyngeal, may be haemorrhagic and even necrotic.~~ Catarrhal accumulations, erosions and the formation of a diphtheritic membrane are often observed in the respiratory tract.Within the urinary tract characteristic echymotic haemorrhages of the epithelial lining of the bladder are often present, especially in bison. ~~while the renal cortex may be affected with multiple raised white foci, each 1–5 mm in diameter and sometimes surrounded by a thin zone of haemorrhage.~~

Histological changes have been the basis for confirming cases of MCF and are characterised by epithelial degeneration, vasculitis, hyperplasia and necrosis of lymphoid organs, and widespread interstitial accumulations of lymphoid cells in nonlymphoid organs. ~~Epithelial lesions may be present at all epithelial surfaces and are characterised by erosion and ulceration, frequently with subepithelial and intraepithelial lymphoid cell infiltration, which may be associated with vasculitis and haemorrhages.~~Vasculitis is generally present and may be pronounced in the brain, affecting veins, arteries, arterioles and venules. It is characterised by lymphoid cell infiltration of the tunica adventitia and media, often associated with fibrinoid degeneration. The brain may also show a nonsuppurative meningoencephalitis with lymphocytic perivascular cuffing and a marked increase in the cellularity of the cerebrospinal fluid. ~~In the lumen there may be 'pavementing' by lymphoid cells, and in severe cases, endothelial damage and subendothelial accumulations by lymphoid cells can sometimes lead to occlusion~~.

Lymph-node hyperplasia is characterised by an expansion of lymphoblastoid cells in the paracortex, while degenerative lesions are generally associated with the follicles. ~~Oedema with lymphoid inflammation often affects the perinodal tissue.~~

The interstitial accumulation of lymphoid cells in nonlymphoid organs, in particular the renal cortex and periportal areas of the liver, is typical, and in the case of the kidney may be very extensive with development of multiple raised white foci, each 1–5 mm in diameter. ~~In the brain there may be a nonsuppurative meningoencephalitis with lymphocytic perivascular cuffing and a marked increase in the cellularity of the cerebrospinal fluid.~~

~~The macroscopic lesions observed in the cornea are reflected histologically by lymphoid cell infiltration originating in the limbus and progressing centrally, with oedema and erosion developing in the more advanced cases. Vasculitis, hypopyon and iridocyclitis also may be present~~.

The pathological features of MCF, irrespective of the agent involved, are essentially similar. However, apart from histological examination, the methods available for diagnosing AlHV-1- and OvHV-2-induced disease tend to be virus-specific and are indicated below for each virus very different and are thus considered separately.

B. DIAGNOSTIC TECHNIQUES

B1. Alcelaphine herpesvirus-1

This form of the disease occurs in the cattle raising regions of eastern Africa where pastoralists use areas grazed by wildebeest, and in southern Africa in areas where wildebeest and cattle are grazed together. The disease, however, can also affect a variety of other ruminant species in zoological collections world-wide and so, apart from antelope of the subfamilies Alcelaphinae and Hippotraginae, it is advisable to regard all ruminants as susceptible.

Sheep associated MCF occurs world-wide in cattle and other species, normally appearing sporadically and affecting only one or a few animals. However, on occasion, incidents occur in which several animals become affected, and this appears to be associated with certain sheep flocks that may continue to transmit disease for a number of years. The disease can also infect and cause substantial losses in North American Bison (*Bison bison*), red deer (*Cervus elaphus*), other deer species and water buffalo (*Bubalus bubalis*) and even more readily in Père David's deer (*Elaphurus davidianus*) and Bali cattle (*Bos javanicus*). OvHV-2 is also responsible for causing MCF in zoological collections, where disease has been reported in a variety of species including giraffe. Disease in pigs has been reported from several countries, but is most frequently recognised in Norway where incidents involving several animals regularly occur.

Disease control: Control at present relies on segregating natural hosts from susceptible species, the extent to which this is enforced depending on the species involved. MCF-affected animals never or rarely transmit infection; hence it is only the natural hosts that can act as a source of infection. Wildebeest are relatively efficient transmitters of infection, and hence their segregation in mixed collections is important. Pastoralists should segregate cattle from wildebeest and pastures recently grazed by them, particularly around the time of wildebeest calving.

With OvHV-2, the requirement to segregate sheep depends on the susceptibility of the species involved. Thus with Père David's deer and Bali cattle, strict separation and avoidance of contact through fomites must be ensured. Equally, every reasonable effort must be taken to segregate bison and farmed deer from sheep, although fallow deer (*Dama dama*) appear to be more resistant to MCF. Cattle are generally managed with sheep without taking precautions to guard against disease transmission.

Zoonotic risk and biosafety requirements: Disease has only been observed in MCF-susceptible species as described above, and only following contact with reservoir host species. The inability of MCF-susceptible species to propagate cell-free virus makes them terminal hosts of the virus. Biosafety requirements should therefore focus on the separation of susceptible hosts from reservoir species, particularly for those considered especially susceptible, such as bison, deer or Bali cattle.

Differential diagnosis: The clinical signs of the 'head and eye' form of MCF resemble those of other diseases that cause oral lesions (Holliman, 2005). Thus BVD/mucosal disease, rinderpest, foot and mouth disease, bluetongue and vesicular stomatitis may be considered as potential differential diagnoses where MCF is suspected. A clear diagnosis of MCF may be supported by additional evidence such as detection of MCF virus DNA, virus-specific antibody response and/or histopathology consistent with MCF.

B. DIAGNOSTIC TECHNIQUES

Table 1. *Test methods available and their purpose*

Method	Purpose			
	Natural host species		Clinically affected animals	All animals
	Population freedom from infection	Individual animal freedom from infection	Confirmation of clinical cases	Prevalence of infection – surveillance
PCR	+	+	+++	++
C-ELISA	+++	+++	++	+++
Virus isolation	+(AlHV-1)	+(AlHV-1)	+(AlHV-1)	+(AlHV-1)
Virus neutralisation	+ (AlHV-1)	+(AlHV-1)	–	–
IFAT	+	+	+	–
Immunoperoxidase test	+	+	+	–

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors limit its application; – = not appropriate for this purpose.
Although not all of the tests listed as category +++ or ++ have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.
PCR = polymerase chain reaction; C-ELISA = competitive inhibition enzyme-linked immunosorbent assay; IFAT = indirect fluorescent antibody tes;. Note that virus isolation and virus neutralisation have only been documented for AlHV-1.

It must be emphasised that the viral cause of SA-MCF cannot be reliably isolated and evidence for OvHV-2 relies on: (a) the presence of antibody in sera of all domestic sheep that cross-reacts with AlHV-1 antigens in the immunofluorescent antibody (IFA) test, enzyme-linked immunosorbent assay (ELISA) and/or immunoblots (Hart *et al*., 2007, Li *et al*., 2001), but not in neutralisation assays; (b) the development of antibody that cross-reacts with AlHV-1 in the IFA test and competitive enzyme-linked immunosorbent assay (C-ELISA) in most cattle with SA-MCF and in experimentally infected ~~hamsters~~ animal models; (c) the detection and cloning of DNA from lymphoblastoid cell lines derived from natural cases of SA-MCF that cross-hybridises with, but is distinct from, AlHV-1 DNA; (d) the detection by PCR of amplicons unique to OvHV-2 in peripheral blood and affected tissues of animals with SA-MCF. More recently, OvHV-2 collected in sheep nasal secretions has been used to infect rabbits, cattle and bison, inducing MCF with characteristic clinical signs and histopathology (Li *et al*., 2011; Taus *et al*., 2006).

Diagnosis of MCF based on clinical signs and gross pathological examination cannot be relied on as these can be extremely variable. Histological examination of a variety of tissues including, by preference, kidney, liver, urinary bladder, buccal epithelium, cornea/conjunctiva and brain, are necessary for reaching a more certain diagnosis. However, detection of antibody to the virus and/or viral DNA can now also be attempted and these are rapidly becoming the methods of choice.

Most laboratory-based tests to detect virus-specific antibody have relied on one attenuated isolate (WC11) that has been subjected to many laboratory passages as a source of viral antigen and DNA (Plowright *et al*., 1960). The full nucleotide sequence of the virulent low passage virus (C500) is now available and will form the basis of further studies of this virus (Ensser *et al*., 1997). Laboratory passage of the AlHV-1 C500 strain leads to attenuation of virulence and the ability to propagate in a cell-free manner, accompanied by genomic changes (Wright *et al*., 2003). This high passage derivative of AlHV-1 C500 has been used as a candidate vaccine for wildebeest-associated MCF and as a source of antigen for serological analysis (Haig *et al*., 2008; Russell *et al*., 2012).

1. Identification of the agent

• Clinically affected animals

a) Isolation

A striking feature of ~~AlHV-1-induced~~ MCF is the lack of detectable viral antigen or herpesvirus-specific cytology within lesions. Confirmation of infection ~~thus relies on~~ by virus recovery can only be performed for AlHV-1 to date, while attempts to recover the disease-causing virus from clinical cases of SA-MCF have failed consistently. However, lymphoblastoid cell lines have been generated from affected cattle and deer, some of which transmit MCF following inoculation into experimental animals (Reid *et al.*, 1989).

Generally, AlHV-1 infectivity is strictly cell associated and thus isolation can be achieved only from cell suspensions either of peripheral blood leukocytes, lymph nodes or other affected tissues. Cell suspensions are prepared in tissue culture fluid, approximately 5×10^6 cells/ml, and inoculated into preformed monolayer cell cultures. Bovine thyroid cells have been used extensively, but most primary and low passage monolayer cell cultures of ruminant origin will probably provide a suitable cell substrate for isolating the virus. Following 36–48 hours' incubation, culture medium should be changed and monolayers should be examined microscopically (×40) for evidence of cytopathic effects (CPE). These appear characteristically as multinucleate foci within the monolayers, which then progressively retract forming dense bodies with cytoplasmic processes that may detach. This is followed by regrowth of normal monolayers. A CPE may take up to 21 days to become visible and is seldom present before day 7. Infectivity at this stage tends to be largely cell associated and thus any further passage or storage must employ methods that ensure that cell viability is retained. Specificity of the isolate should be determined using specific antisera or monoclonal antibodies (MAbs) in fluorescence or immunocytochemical tests.

b) Viral DNA

Characteristically, very little viral DNA can be detected within affected tissues, hence it is necessary to amplify the viral genome either by conventional culture or the polymerase chain reaction (PCR).

The full sequence of the C500 isolate of AlHV-1 and of two isolates of OvHV-2 have been published, permitting the design of primers for PCR reactions from conserved regions of the genome (Ensser *et al.* 1997; Hart *et al.*, 2007; Taus *et al*., 2007). Nested and real-time PCR assays have been developed for AlHV-1 and OvHV-2 (Baxter *et al*., 1993; Flach *et al*., 2002; Hussy *et al*., 2001; Traul *et al*., 2005) while a pan-herpesvirus PCR (VanDevanter *et al*., 1996) has been used to identify CpHV-2 in Sika deer with MCF (Foyle *et al*., 2009) and a virus associated with MCF in White-tailed deer (Li *et al*., 2000). This assay targets the viral DNA polymerase gene sequence and has been employed for phylogenetic comparison of AlHV-1 and related viruses (Li *et al*., 2005a).

- **PCR protocols**

These protocols are based on published nested PCR assays designed to detect OvHV-2 (Baxter *et al*., 1993) or to distinguish AlHV-1 and OvHV-2 (Flach *et al*., 2002) in DNA samples from natural hosts or MCF-affected species. DNA purification methods should avoid organic extraction as this can leave residues that inhibit PCR. Silica-based genomic DNA extraction methods have been extensively used and appear reliable. Methods for extraction of DNA from fixed tissue samples should be validated before use in these assays. An example protocol is given below, however optimal reaction conditions should be validated for each system of enzymes and buffers. Protocols for real-time PCR to detect MCF virus DNA (Traul *et al*., 2005; Hussy *et al*., 2001) are not given as these should be optimised for each reagent set and analysis system used.

- **Protocol 1: Hemi-nested PCR to detect OvHV-2 DNA (Baxter *et al*., 1993)**

Primers

Name	Length	Sequence
556	30 mer	5'-AGT CTG GGT ATA TGA ATC CAG ATG GCT CTC-3'
555	28 mer	5'-TTC TGG GGT AGT GGC GAG CGA AGG CTTC-3'
755	30 mer	5'-AAG ATA AGC ACC AGT TAT GCA TCT GAT AAA-3'

Primary amplification (*product size 422 bp*)

Prior to PCR, a master mix is made up, comprising all components except template DNA. This is then dispensed into PCR tubes containing test or control DNA. This approach minimises pipetting errors when assaying large numbers of samples. The master mix comprises (per reaction): 10× PCR buffer, 5 µl; $MgCl_2$ (25 mM), 1 µl; dNTP mix (1 mM), 5 µl; primer 556 (10 µM), 1 µl; primer 755 (10 µM), 1 µl; Taq DNA polymerase (5 u/µl), 0.125 µl; and nuclease-free water, 31.875 µl; making a total of 45 µl per reaction. Samples of 5 µl, containing up to 1 µg of test or control DNA, are placed in PCR tubes and 45 µl of master mix are added to each tube. The tubes are then used for PCR according to the following protocol, using a PCR machine with heated lid. To use a PCR machine without a heated lid, mineral oil should be overlaid on each PCR reaction to prevent evaporation.

Suggested cycling conditions are: Hot-start activation at 95°C for 15 minutes; followed by 15 cycles of 94°C for 60 seconds, 60°C for 60 seconds and 72°C for 60 seconds; with a final extension at 72°C for 10 minutes. The conditions should be adjusted according to the Taq polymerase and the thermocycler used.

Secondary amplification (*product size 238 bp*)

The master mix for the secondary amplification comprises (per reaction): 10× PCR buffer, 5 µl; $MgCl_2$ (25 mM), 1 µl; dNTP mix (1 mM), 5 µl; primer 556 (10 µM), 1 µl; primer 555 (10 µM), 1 µl; Taq DNA polymerase (5 u/µl), 0.125 µl; and nuclease-free water, 33.875 µl; making a total of 48 µl. Samples of 2 µl of each primary amplification product are placed in PCR tubes and 48 µl of master mix are added to each tube. Cycling conditions for the secondary PCR are the same as for the primary PCR, except that 30 cycles of amplification are used. After amplification approximately 10 µl of each secondary PCR reaction should be run on a 1.8 % agarose gel to visualise the PCR products.

- **Protocol 2: Hemi-nested PCR to distinguish AlHV-1 and OvHV-2 DNA (Flach *et al.*, 2002)**

Primers

Name	Length	Sequence*
Primer POL1	24-mer	5'-GGC (CT)CA (CT)AA (CT)CT ATG CTA CTC CAC-3'
Primer POL2	21-mer	5'-ATT (AG)TC CAC AAA CTG TTT TGT-3'
Primer OHVPol	20-mer	5'-AAA AAC TCA GGG CCA TTC TG-3'
Primer AHVPol	20-mer	5'-CCA AAA TGA AGA CCA TCT TA-3'

*base positions in parentheses are degenerate – the oligonucleotide will contain either of the two indicated bases at these positions.

The primers POL1 and POL2 target a segment of the DNA polymerase gene which is conserved in both OvHV-2 and AlHV-1, amplifying a fragment of 386bp. OHVPol and AHVPol are specific primers for OvHV-2 and AlHV-1 respectively, which amplify 172bp products.

Primary amplification

Master mix, per reaction: 10× buffer, 2.5 µl; $MgCl_2$ (25 mM), 0.5 µl; dNTP mix (1 mM), 2.5 µl; primer POL1 (10 µM), 1 µl; primer POL2 (10 µM), 1 µl; Taq DNA polymerase (5 u/µl), 0.125 µl; nuclease-free water, 12.375 µl (to 25 µl). Samples of 5 µl, containing up to 1 µg of test or control DNA, are placed in PCR tubes and 20 µl of master mix are added to each tube. The tubes are then used for PCR according to the following protocol: Hot-start activation at 95°C for 15 minutes; followed by 25 cycles of 94°C for 60 seconds, 60°C for 60 seconds and 72°C for 60 seconds; with a final extension at 72°C for 10 minutes. The conditions should be adjusted according to the Taq polymerase and the thermocycler used.

Secondary amplification

Master mix, per reaction: 10× buffer, 2.5 µl; $MgCl_2$ (25 mM), 0.5 µl; dNTP mix (1 mM), 2.5 µl; primer AHVpol or OHVpol (10 µM), 1 µl; primer POL2 (10 µM), 1 µl; Taq DNA polymerase (5 u/µl), 0.125 µl; nuclease-free water, 12.375 µl (to 25 µl). Samples of 2 µl of each primary amplification product are placed in PCR tubes and 23 µl of master mix are added to each tube. Cycling conditions for the secondary PCR are the same as for the primary PCR, except that 30 cycles of amplification are used. After amplification approximately 10 µl of each secondary PCR reaction should be run on a 1.8% agarose gel.

- **Natural hosts**

It is almost certain that all free-living wildebeest are infected with AlHV-1 by 6 months of age, virus having been spread ~~as an intense epizootic~~ intensively during the perinatal period. The species *Connochaetes taurinus taurinus*, *C.t. albojubatus* and *C. gnu* are all assumed to be infected with the same virus. Infection also appears to persist in most groups of wildebeest held in zoological collections. However, it is possible that infection may be absent in animals that have been isolated during calf-hood or that live in small groups. Natural infection has been successfully demonstrated by *in-situ* hybridisation on lung sections from *C.t. taurinus* calves in South Africa (Michel *et al*., 1997).

Following infection there is a brief period when virus is excreted in a cell-free form and can be isolated from nasal swabs. Virus can also be isolated from blood leukocytes at this time, but in older animals this is less likely to be successful unless the animal is immunosuppressed either through stress or pharmacological intervention. In

addition, virus may be isolated by establishing cultures of tissues from apparently normal animals, and this has been achieved in monolayer cultures of both kidney and thyroid cells from adult animals.

The domestic sheep is the natural host of OvHV-2 and probably all sheep populations are infected with the virus in the absence of any clinical response. Studies of the dynamics of infection within sheep flocks have however, generated conflicting results with some suggesting productive infection occurs in the first weeks of a lamb's life while others suggest infection of most lambs does not occur until 3 months of age with excretion of infectious virus occurring between 5 and 6 months (Li *et al*., 2004). There is also evidence that some lambs may become infected *in utero* while other studies suggest that removal of lambs from their dams during the first week permits the establishment of virus-free animals. There may therefore be considerable variation in the dynamics of infection in different flocks. However, circumstantial evidence of the occurrence of MCF in susceptible species does suggest that the perinatal sheep flock is the principal source of infection, but that periodic recrudescence of infection may occur in sheep of all ages.

In addition to domestic sheep, domestic goats and other members of the subfamily *Caprinae* have antibody that reacts with AlHV-1 in a similar pattern to sheep serum. This implies that these species are infected with viruses similar to OvHV-2. Some goats have been found to be OvHV-2 positive by PCR, while a few cases of MCF caused by CpHV-2 have been reported (Foyle *et al*., 2009). Other large antelope of the subfamilies *Alcelaphinae* and *Hippotraginae* are also infected with antigenically closely related gammaherpesviruses (Li *et al*., 2005a), but there is no evidence that they can spread to other species and cause MCF, except rarely in captive populations.

2. Serological tests

• Clinically affected animals

The antibody response of clinically affected animals is limited, with no neutralising antibody developing. Antibody to OvHV-2 has only been detected using AlHV-1 as the source of antigen. Antibody to AlHV-1 can be detected in 70–80% of clinically affected cattle by IFA or immunoperoxidase test (IPT) procedures, but may not be present in affected deer or animals that develop acute or peracute disease. ~~Antibody in clinical cases can be demonstrated consistently by immunofluorescence or the immunoperoxidase test (IPT) using WC11-infected cell cultures as substrate.~~ A ~~competitive inhibition~~ C-ELISA was first developed for detecting antibody to OvHV-2 (Li *et al*., 1994) using an MAb (15-A) that targets an epitope that appears to be conserved among all MCF viruses and is probably also applicable to AlHV-1 infected animals (Li *et al*., 2005a). The test has been employed to detect antibody in serum of wild and domestic ruminants in North America and appears to have some merit (Section B.2.d) (Li *et al*., 2001). A comparative study of MCF diagnosis by histopathology, C-ELISA and OvHV-2-specific PCR showed that most cattle classified as MCF-positive by histopathology also had detectable MCF virus-specific antibodies and OvHV-2 DNA in the blood (Muller-Doblies *et al*., 1998). A direct ELISA based on WC11 antigens has also been developed and assay results correlated well with parallel tests using the C-ELISA (Fraser *et al*. 2006).

• Natural hosts

Antibody appears to develop consistently in wildebeest following infection and can be identified by neutralisation assays using the cell-free isolate WC11, or by immunofluorescence, again using the WC11 isolate and anti-bovine IgG, which has been shown to react with wildebeest IgG. The Minnesota MCF virus strain, which is indistinguishable from the WC11 strain of AlHV-1, is used for C-ELISA antigen production.

Antibody to OvHV-2 has only been detected using AlHV-1 as the source of antigen. In a study on the reaction of sheep serum to the structural proteins of AlHV-1 in immunoblots, the reactivity of different sera varied strikingly, indicating that individual sheep responded differently with regard to antibody recognition of cross-reacting epitopes of AlHV-1.

There has been no attempt so far to standardise the IFA test and the IPT, but the two methods below are given as examples. The C-ELISA is available as a commercial kit.

a) Indirect fluorescent antibody test

The IFA is less specific than virus neutralisation (VN); it can be used to demonstrate several varieties of 'early' and 'late' antigens in AlHV-1-infected cell monolayers. Antibodies reacting in the IFA test or the IPT develop in cattle and experimentally infected rabbits during the incubation period, and later in the clinical course of the disease, though cross-reactions with some other bovine herpesviruses, as well as OvHV-2, reduce the differential diagnostic value. Detection of such cross-reacting antibodies can sometimes be useful in supporting a diagnosis of SA-MCF.

• Preparation of fixed slides

Inoculate nearly or newly confluent cell cultures ~~(see Section B1.2.c)~~ with AlHV-1 (strain WC11). Uninoculated control cultures should be processed in parallel. At about 4 days – when the first signs of CPE

are expected to appear but before overt CPE is visible – treat the cultures as follows: discard the medium, wash with PBS, remove the cells with trypsin–versene solution, spin down cells at approximate 800 ***g*** for 5 minutes, discard the supernatant fluid, and resuspend the cells in 10 ml of phosphate buffered saline (PBS) for each 800 ml plastic bottle of cell culture.

Make test spots of the cell suspension on two wells of a polytetrafluoroethylene-coated multiwell slide; air-dry and fix in acetone. Stain the spots with positive standard serum and conjugated anti-IgG to the appropriate species. Examine the incidence of positive and negative cells under a fluorescence microscope. Adjust the cell suspension by adding noninfected cells and/or PBS to give a suitable concentration that will form a single layer of cells when spotted on to the slide, with clearly defined positive cells among a background of negative cells.

Spot the adjusted positive cell suspension and the control negative suspensions on to multiwell slides in the desired pattern, and air-dry. Fix in acetone for 10 minutes. Rinse, dry and store over silica gel in a sealed container at –70°C.

An alternative procedure, which is easier to evaluate, is to prepare monolayers of infected and noninfected cells in Leighton tubes or chamber slides. The cell monolayers are infected with from 150 to 200 $TCID_{50}$ (50% tissue culture infective dose) of virus that has been diluted in cell culture medium. The infected and noninfected slides are fixed in acetone and stored, as above, at –70°C.

- **Test procedure**

i) Rehydrate the slides for 5 minutes with PBS, rinse in distilled water and air-dry.

ii) Dilute sera 1/20 in PBS. Samples that give high background staining may be retested at higher dilutions. Apply diluted fluids to one MCF virus-positive cell spot and one negative control spot for each sample. Include positive and negative serum controls. Ideally, the test should be validated by titrating the control positive to determine its end-point.

iii) Incubate at 37°C for 30 minutes in a humid chamber.

iv) Drain the fluids from the spots. Wash the slides in two changes of PBS, for 5 minutes each.

v) Wash in PBS for 1 hour with stirring, and then air-dry the slides.

vi) Apply rabbit anti-bovine IgG fluorescein isothiocyanate (FITC) conjugate at a predetermined working dilution.

vii) Incubate at 37°C for 20 minutes, drain the slides, and wash twice in PBS for 10 minutes each.

viii) Counterstain in Evans blue 1/100,000 for 30 seconds, and wash with PBS for 2 minutes. Dip in distilled water, dry and mount in PBS/glycerol (50/50).

ix) Examine by fluorescence microscopy for specific binding of antibody to the infected cells.

b) Immunoperoxidase test

A dilution of bovine turbinate (BT) cell-cultured AlHV-1 containing approximately 10^3 $TCID_{50}$ is made in a freshly trypsinised suspension of BT cells and seeded into Leighton tubes containing glass cover-slips, 1.6 ml per tube, or four-chambered slides, 1.0 ml per chamber.

Observe the cell cultures at 4–6 days for CPE and fix the cultures with acetone when signs of CPE begin. Remove the plastic chambers, but not the gaskets, from the slide chambers before fixation, and use acetone (e.g. UltimAR grade) that will not degrade the gasket. Store the fixed cells at –70°C.

- **Test procedure**

i) Prepare IPT diluent (21.0 g NaCl and 0.5 ml Tween 20 added to 1 litre of 0.01 M PBS, pH 7.2) and washing fluid (0.5 ml Tween 20 added to 1 litre of 0.01 M PBS, pH 7.2).

ii) Dilute the serum to be tested 1/20 in IPT diluent and overlay 150–200 µl on to a fixed virus-infected cover-slip or slide chamber.

iii) Incubate the cover-slip in a humid chamber at 37°C for 30 minutes.

iv) Dip the cover-slip three times in washing fluid.

v) Overlay 150–200 µl of diluted (1/5000 in IPT diluent) peroxidase-labelled anti-bovine IgG on to the cover-slip or slide chamber.

vi) Incubate the cover-slip or slide chamber in a humid chamber at 37°C for 30 minutes.

vii) Dip the cover-slip three times in washing fluid.

viii) Dilute the AEC substrate (3-amino-9-ethylcarbazole, 20 mg/ml in dimethyl formamide) in distilled water (5 ml of distilled water, 2 drops 50 mM sodium acetate buffer pH 5.0, 2 drops hydrogen peroxide (30%), and 3 drops AEC) and apply to the cover-slip or slide chamber.

ix) Incubate in a humid chamber at 37°C for 8–10 minutes.

x) Dip the cover-slip in distilled water, air-dry, and mount on a glass slide. Slide chambers are read dry.

xi) The slide is read on a light microscope. The presence of a reddish-brown colour in the nuclei of the infected cells indicates a positive reaction.

c) Virus neutralisation

Tests have been developed for detecting antibodies to AlHV-1 in both naturally infected reservoir and indicator hosts. The first of these is a VN test using cell-free virus of the WC11 strain, and another uses a hartebeest isolate (AlHV-2). The attenuated strain of AlHV-1 C500 may also be used. ~~AlHV-1 and AlHV-2~~ These viruses have cross-reactive antigens and therefore either strain can be used in the test. The test is laborious, but can be performed in microtitre plates using low passage cells or cell lines. The main applications have been in studying the range and extent of natural gammaherpes virus infection in wildlife, captive species in zoos and, to a lesser extent, sheep populations. It has also been useful in attempts to develop vaccines, ~~all of which have had limited success~~ including the recent AlHV-1 vaccine that induced neutralising antibodies in cattle blood plasma and nasal secretions (Haig *et al*., 2008). The VN test is of no value as a diagnostic test in clinically affected animals as no VN antibody develops in clinically susceptible species.

AlHV-1 stock (e.g. strain WC11) is grown in primary or secondary cell cultures of bovine kidney, bovine thyroid, low passage bovine testis, or another permissive cell type. The virus is stored in aliquots at –70°C. The stock is titrated to determine the dilution that will give 100 $TCID_{50}$ in 25 µl under the conditions of the test.

- **Test procedure**

i) Inactivate the sera for 30 minutes in a water bath at 56°C.

ii) Make doubling dilutions of test sera in cell culture medium from 1/2 to 1/16 using a 96-well flat-bottomed cell-culture grade microtitre plate, four wells per dilution and 25 µl volumes per well. Positive and negative control sera are also included in the test. No standard sera are available, but internal positive standards should be prepared and titrated in an appropriate range.

iii) Add 25 µl per well of AlHV-1 ~~WC11~~ virus stock at a dilution in culture medium calculated to provide 100 $TCID_{50}$ per well.

iv) Incubate for 1 hour at 37°C. The residual virus stock is also incubated.

v) Back titrate the residual virus in four tenfold dilution steps, using 25 µl per well and at least four wells per dilution.

vi) Add 50 µl per well of bovine kidney cell suspension at 3×10^5 cells/ml.

vii) Incubate the plates in a humidified CO_2 atmosphere at 37°C for 7–10 days.

viii) Read the plates microscopically for CPE. Validate the test by checking the back titration of virus (which should give a value of 100 $TCID_{50}$ with a permissible range 30–300) and the control sera. The standard positive serum should give a titre within 0.3 $\log_{10}$ units of its predetermined mean.

ix) The test serum results are determined by the Spearman–Kärber method as the dilution of serum that neutralised the virus in 50% of the wells.

x) A negative serum should give no neutralisation at the lowest dilution tested (1/2 equivalent to a dilution of 1/4 at the neutralisation stage).

d) Competitive inhibition enzyme-linked immunosorbent assay (C-ELISA)

A C-ELISA was ~~first~~ developed for detecting antibody to OvHV-2 (Li *et al*., 1994; 2001) using an MAb (15-A) that targets an epitope on a complex of glycoproteins that appears to be conserved among all MCF viruses. The MAb was raised against the Minnesota isolate of virus, which is indistinguishable from the WC11 strain of AlHV-1. The test has been employed to detect antibody in serum of wild and domestic ruminants in North America and antibody to the following pathogenic viruses has been detected: AlHV-1, AlHV-2, OvHV-2, CpHV-2 and the herpesvirus of unknown origin observed to cause classic MCF in white-tailed deer, as well as the MCF-group viruses not yet reported to be pathogenic, such as those carried by the oryx, muskox, and others (Li *et al*. 2005a). ~~The test has recently beenwas reformatted to increase sensitivity (Hsu *et al*., 1990). This change was made to enable the detection of antibody in newly infected lambs and animals in the acute~~

~~stage of the disease, which were sometimes not detected in the previous format.~~ The C-ELISA has the advantage of being faster and more efficient than the IFA or IPT. Additional validation data will become available as its use is expanded to more laboratories in other parts of the world. One comparison between C-ELISA, PCR and histopathological diagnosis of SA-MCF (Muller-Doblies *et al*., 1998) suggested that the three approaches had good concordance.

The complete reagent set for the C-ELISA, including pre-coated plates, labelled MAb and control sera, is commercially available. For laboratories wishing to prepare their own antigen-coated plates, the following protocol is provided. ~~Immuno 4~~ ELISA plates ~~(Dynatech Lab, Chantilly, Virginia)~~ are coated at 4°C (39°F) for 18–20 hours with 50 µl of a solution containing 0.2 µg per well of semi-purified MCF viral antigens (Minnesota or WC11 isolates of AlHV-1) in 50 mM carbonate/bicarbonate buffer (pH 9.0). The coated plates are blocked at room temperature (21–25°C, 70–77°F) for 2 hours with 0.05 M PBS containing 2% sucrose, 0.1 M glycine, 0.5% bovine serum albumin and 0.44% NaCl (pH 7.2). After blocking, wells are emptied and the plates are then dried in a low humidity environment at 37°C for 18 hours, sealed in plastic bags with desiccant, and stored at 4°C (39°F) (Li *et al*., 2001). MAb 15-A is conjugated with horseradish peroxidase ~~by the VMRD, Inc.~~ using a standard periodate method.

- **Test procedure**

i) Dilute positive and negative controls and test samples (either serum or plasma) 1/5 with dilution buffer (PBS containing 0.1% Tween 20, pH 7.2).

ii) Add 50 µl of diluted test or control samples to the antigen-coated plate (four wells for negative control and two wells for positive control). Leave well A1 empty and for use as a blank for the plate reader.

iii) Cover the plate with parafilm and incubate for 60 minutes at room temperature, (21–25°C, 70–77°F).

iv) Using a wash bottle, wash the plate three times with wash buffer (same as dilution buffer: PBS containing 0.1% Tween 20, pH 7.2).

v) Prepare fresh 1 × antibody-peroxidase conjugate in dilution buffer according to previous titration/optimisation for each conjugate preparation, or to the manufacturer's instructions. ~~by diluting one part of the 100 × conjugate with 99 parts of dilution buffer.~~

vi) Add 50 µl of diluted antibody-peroxidase conjugate to each sample well. Cover the plate with parafilm and incubate for 60 minutes at room temperature (21–25°C, 70–77°F).

vii) Wash the plate with wash buffer three times.

viii) Add 100 µl of tetramethylbenzidine substrate solution ~~(TMB Microwell, BioFX Laboratories, Owings Mills, Maryland)~~ to each sample well. Incubate for 60 minutes at room temperature (21–25°C; 70–77°F). Do not remove the solution from the wells.

ix) Add 100 µl of stop solution (0.18 M sulphuric acid) to each well. Do not remove the solution from the wells.

x) Read the optical densities (OD) on an ELISA plate reader at 450 nm.

xi) Calculating % inhibition:

$$100 - \frac{\text{Sample OD (Average)} \times 100}{\text{Mean negative control OD}} = \%\text{inhibition}$$

xii) *Interpreting the results:* If a test sample yields equal to or greater than 25% inhibition, it is considered positive. If a test sample yields less than 25% inhibition, it is considered negative.

xiii) *Test validation:* The mean OD of the negative control must fall between 0.40 and 2.10. The mean of the positive control must yield greater than 25% inhibition.

~~B2. Ovine herpesvirus 2~~

~~This form of the disease occurs world wide in cattle and other species, normally appearing sporadically and affecting only one or a few animals. However, on occasion, incidents occur in which several animals become affected, and this appears to be associated with certain sheep flocks that may continue to transmit disease for a number of years. The disease can also spread and cause substantial losses in North American Bison (*Bison bison*), red deer (*Cervus elaphus*), other deer species and water buffalo (*Bubalus bubalis*) and even more readily to Père David's deer (*Elaphurus davidianus*) and Bali cattle (*Bos javanicus*). OvHV-2 is also responsible for causing MCF in zoological collections, where disease has been reported in a variety of species including giraffe. Disease in pigs has been reported from several countries, but is most frequently recognised in Norway where incidents involving several animals regularly occur.~~

Diagnosis based on clinical signs and gross pathological examination cannot be relied on as these can be extremely variable. Histological examination of a variety of tissues including, by preference, kidney, liver, urinary bladder, buccal epithelium, cornea/conjunctiva and brain, has been the only method of reaching a more certain diagnosis. However, detection of antibody to the virus and/or viral DNA can now also be attempted and are rapidly becoming the methods of choice.

It must be emphasised that the viral cause of SA-MCF cannot be reliably isolated and evidence for OvHV-2 relies on: (a) the presence of antibody in sera of all domestic sheep that cross reacts with AlHVA/HV-1 antigens in the IFA test and immunoblots (Hart *et al*., 2007), but not in neutralisation assays; (b) the development of antibody that cross-reacts with AlHVA/HV-1 in the IFA test and CI ELISA in most cattle with SA-MCF and in all experimentally infected hamsters: (c) the detection and cloning of DNA from lymphoblastoid cell lines derived from natural cases of SA-MCF that cross hybridises with, but is distinct from, AlHVA/HV-1 DNA; (d) the detection by PCR of amplicons unique to OvHV-2 in peripheral blood and affected tissues.

1. Identification of the agent

• Clinically affected animals

Attempts to recover the disease causing virus from clinical cases have failed consistently. There are, however, several reports of the recovery of different viral agents from clinical cases, none of which has established any causal relationship; their isolation is certainly fortuitous or due to laboratory contamination. However, lymphoblastoid cell lines have been generated from affected cattle and deer, some of which transmit MCF following inoculation into experimental animals (Reid *et al*., 1989). Such cell lines contain viral sequences that hybridise with clones of AlHVA/HV-1 DNA (Bridgen & Reid, 1991). A virus sequence was cloned from such a cell line that coded for a tegument protein that was distinct from AlHV-1. Subsequently the whole length of the viral genome has been cloned and the nucleotide sequence determined (Hart *et al*., 2007). Primers were identified within this sequence that were suitable for use in the PCR, and a sensitive protocol was designed in which a fragment of 422 base pairs (bp) is amplified initially, followed by amplification of a truncated internal fragment of 238 bp. It has been proven that this test is able to detect as few as 35 viral genome equivalents and that no product is amplified from AlHVA/HV-1 or other bovid herpesviruses (Baxter *et al*., 1993). This PCR is thus both highly specific and sensitive for OvHV-2 and has been employed world wide in studies of the disease in clinically affected animals and the natural host. It is emerging as a robust test that can be employed to detect viral DNA in peripheral blood leukocytes of clinically affected animals as well as fresh tissues and paraffin embedded samples collected at post mortem. The use of magnetic particles to purify DNA prior to amplification has been reported to be an additional improvement to the test, but is yet to be evaluated. A quantitative fluorogenic PCR assay for OvHV-2 has also been established and validated using the semi-nested PCR (Baxter *et al*., 1993) as a gold standard (Hussy *et al*., 2001) and is likely to have valuable future application.

While early studies indicated that infection of MCF-susceptible species would normally result in death, some prospective studies in high incidence herds of animals suggest that animals may become infected without developing a clinical response. Factors that predispose animals to infection and development of disease are not understood and it is likely to be a complex interaction of environmental, host factors and the infecting virus. That MHC class 11a polymorphism may contribute to resistance of American bison as suggested in one study (Traul *et al*., 2007) is of interest and should be further examined.

• Polymerase chain reaction

Extraction of DNA from clinical material is performed according to the protocol defined in an appropriate extraction kit (e.g. Quiagen DNeasy Tissue Kit). Amplification reactions are performed in 50 µl volumes containing not more than 2 µg test DNA in 10 mM Tris HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.01% (v/v) gelatine, 10% (v/v) dimethyl sulfoxide (DMSO), 200 µm dATP, dCTP, dGTP and dTTP (Pharmacia), 1 µM of each primer and 2 units Taq DNA polymerase overlaid with 50 µl mineral oil (Sigma) to prevent evaporation.

The programme consists of a precycle at 99°C for 3 minutes, after which dNTP and enzyme mix are added. This is followed by 25 cycles of 94°C for 20 seconds, 60°C for 30 seconds and 72°C for 30 seconds. A 2 µl aliquot of the primary amplification product, specified by the primer pair 556/755, is transferred directly to a new reaction mixture and amplified using the primer pairs 556/555 under identical conditions for a further 25 cycles with a final extension at 72°C for 5 minutes.

Final amplification products (10 µl) are analysed directly by 1.8% agarose gel electrophoresis and ethidium bromide fluorescence. With each batch of test samples a known positive control and distilled water are also amplified and analysed.

• Natural hosts

The domestic sheep is the natural host of OvHV-2 and probably all sheep populations are infected with the virus in the absence of any clinical response. Studies of the dynamics of infection within sheep flocks have however, generated conflicting results with some suggesting productive infection occurs in the first weeks of a lamb's life while others suggest infection of most lambs does not occur until 3 months of age with excretion of infectious virus occurring between 5 and 6 months (Li *et al*., 2004). There is also evidence that some lambs may become infected *in utero* while other studies suggest that removal of lambs from their dams during the first week permits the establishment of virus free animals. There may therefore be considerable variation in the dynamics of infection in different flocks. However, circumstantial evidence of the occurrence of MCF in susceptible species does suggest that the perinatal sheep flock is the principal source of infection, but that periodic recrudescence of infection may occur in sheep of all ages.

Factors that predispose to virus shedding and transmission to MCF susceptible hosts remain speculative.

In addition to domestic sheep, domestic goats and other members of the subfamily Caprinae have antibody that reacts with AlHVA/HV-1 in a similar pattern to sheep serum. This implies that these species are infected with viruses similar to OvHV-2, and some goats have been found to be positive to an OvHV-2 PCR, though their potential role in causing MCF would appear to be very limited.

2. Serological tests

Antibody to OvHV-2 has only been detected using AlHVA/HV-1 as the source of antigen. Antibody to AlHVA/HV-1 can be detected in 70–80% of clinically affected cattle by IFA or IPT procedures, but may not be present in affected deer or animals that develop acute or peracute disease. Antibody is detected by IFA using tissue culture cells infected with AlHVA/HV-1. Cell monolayers grown on cover slips exhibiting 10–50% CPE are harvested, washed, fixed in acetone and used in the assay. Cover-slips are mounted with DPX, the side containing the cells facing uppermost, on microscope slides and treated with 10% normal horse serum before progressing with a conventional IFA test. The IPT procedure can be carried out as for AlHVA/HV-1. The only virus of cattle that has been reported to cross react with AlHVA/HV-1 is bovine herpesvirus 4 (BHV-4). Thus the negative control for this test should be similarly infected monolayers of BHV-4. Sera are only considered to be positive when foci show characteristic intranuclear distribution of antigen with little or no cytoplasmic staining being detected in the AlHVA/HV-1 infected cells and no reaction in the BHV-4 infected cells. Sera that react to antigens of both viruses are considered to be inconclusive. A CI ELISA has been developed for detecting antibody to OvHV-2 (Li *et al*., 1994) using a MAb (15-A) raised against the so called Minnesota isolate of virus, which is indistinguishable from AlHVA/HV-1. The test has been employed to detect antibody in serum of wild and domestic ruminants in North America and appears to have some merit (Section B1.2.d) (Li *et al*., 2001). In a study on the reaction of sheep serum to the structural proteins of AlHVA/HV-1 in immunoblots, the reactivity of different sera varied strikingly, indicating that individual sheep responded differently with regard to antibody recognition of cross-reacting epitopes of AlHVA/HV-1.

B3. Control

Control at present relies on segregating natural hosts from susceptible species, the extent to which this is enforced depending on the species involved. With AlHV-1, it would appear that MCF affected animals never or rarely transmit infection; hence it is only the natural hosts that can act as a source of infection. Wildebeest would appear to be relatively efficient transmitters of infection to most other categories of ruminant, and hence their segregation in mixed collections is important. Likewise, pastoralists must ensure that cattle are entirely segregated from the vicinity of wildebeest and pastures recently grazed by them, particularly around the time of wildebeest calving.

With OvHV-2, the requirement to segregate sheep depends on the susceptibility of the species involved. Thus with Père David's deer and Bali cattle, strict separation and avoidance of contact through fomites must be ensured. Equally, with bison and farmed deer every reasonable effort must be taken to segregate the management of sheep, although fallow deer (*Dama dama*) appear to be more resistant to MCF. Cattle only rarely develop SA-MCF, and thus are generally managed with sheep without taking precautions to guard against disease transmission. However, if multiple cases do occur, it is essential to segregate the sheep flock as far as possible from cattle. As such flocks may continue to be sources of infection for some years, disposal of these flocks for slaughter should be considered.

Virus also appears to have been transmitted over substantial distances thus it is not possible to define the distance that sheep should be segregated.

The possibility that very long incubation periods may occur, up to 9 months, further necessitates a guarded prognosis when advising on the control of such outbreaks.

C. REQUIREMENTS FOR VACCINES

At present no vaccine has been licensed for this disease.

Vaccination against MCF could be considered for use in those farmed species that have higher exposure or susceptibility to MCF, such as cattle in regions of East and South Africa where breeding wildebeest are prevalent, Bali cattle, bison in North America, farmed deer worldwide and susceptible species in zoological collections. Vaccination of reservoir hosts, such as wildebeest or sheep, is unlikely to be commercially viable and this is also the case for the majority of cattle herds that are at risk of sporadic SA-MCF. Numerous attempts to produce a protective vaccine against the AlHV-1 form of the disease have met with disappointing results. However, recent trials that focussed on stimulating high titres of neutralising antibody in nasal secretions of cattle have produced encouraging results (Haig *et al*., 2008). This live attenuated vaccine induced protection against intranasal experimental challenge with pathogenic AlHV-1. Protection was also found to persist for at least 6 months (Russell *et al*., 2012). This approach is likely to be the target for further research, including field trials.

As OvHV-2 cannot be successfully propagated in the laboratory no attempts at developing a vaccine have been attempted. However, recent work to develop a cattle challenge system for OvHV-2 using virus from sheep nasal secretions (Taus *et al* 2006) makes the testing of OvHV-2 vaccine candidates a possibility.

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CHAPTER 2.4.18.

TRYPANOSOMOSIS
(tsetse-transmitted)

SUMMARY

Definition of the disease: *Tsetse-transmitted trypanosomosis[1] is a disease complex caused by several species of protozoan parasites of the genus* Trypanosoma*, mainly transmitted cyclically by the genus* Glossina (*tsetse flies*), *but also transmitted mechanically by several biting flies* (*tabanids, stomoxes, etc.*). *The disease can affect various species of mammals but, from an economic point of view, tsetse-transmitted trypanosomosis, is particularly important in cattle. It is mainly caused by* Trypanosoma congolense*,* T. vivax *and, to a lesser extent,* T. brucei brucei*.*

Description of disease: *Tsetse-transmitted trypanosomosis is a classically acute or chronic disease that causes intermittent fever and is accompanied by anaemia, oedema, lacrimation, enlarged lymph nodes, abortion, decreased fertility, loss of appetite and weight, leading to early death in acute forms or to digestive and/or nervous signs with emaciation and eventually death in chronic forms.*

Identification of the agent: *Several parasite detection techniques can be used, including the microscopic examination of the wet ~~and~~ or dry-stained thick or thin blood films. Diagnostic sensitivity is increased significantly by concentrating the parasites prior to examination in combination with a phase-contrast or dark-ground microscope. The centrifugation parasite concentration techniques have the added advantage that the packed cell volume, and hence the level of anaemia, can be determined at the individual animal and/or herd level. A highly specific and more sensitive test, used in an increasing number of laboratories, is the polymerase chain reaction, which can identify parasites at the genus, species or subspecies level, depending on the cases.*

Serological tests: *Two trypanosomal antibody detection tests, the indirect fluorescent antibody test and the antibody-detection enzyme-linked immunosorbent assay* (*ELISA*)*, are routinely used for the detection of antibodies in cattle. They have high sensitivity and genus-specificity but can only be used for the presumptive diagnosis of trypanosomosis. The antibody-detection ELISA, in particular, lends itself to automation and will allow a high degree of standardisation when recombinant antigens have been developed and validated. However, they are at the present time carried out with native soluble antigens of trypanosomes grown in rodents with satisfying sensitivity and specificity.*

Requirements for vaccines ~~and diagnostic biologicals~~: *No vaccines are in use at the present time. ~~Diagnostic biological products produced from cultured parasites are available and in use for the processing of the IFAT and the indirect ELISA.~~*

A. INTRODUCTION

Trypanosomes are flagellate protozoans that inhabit the blood plasma, the lymph and various tissues of their hosts. The genus *Trypanosoma* belongs to the protozoan branch, order Kinetoplastida, family Trypanosomatidae.

~~1 Note on nomenclature of parasitic diseases:~~
~~The World Association for Advances in Veterinary Parasitology has recommended a 'Standardised Nomenclature of Animal Parasitic Diseases' (Kassai T., Cordero del Campillo M., Euzeby J., Gaafar S., Hiepe Th. & Himonas C.A. [1988]. Vet. Parasitol., **29,** 299–326). In principle, the disease name is constructed by adding the suffix '-osis' to the stem of the name of the parasite taxon. This terminology has been followed in this Terrestrial Manual, and 'trypanosomosis' therefore replaces the old term of 'trypanosomiasis'.~~

Tsetse-transmitted trypanosomes belong to the salivarian section, subgenus Nannomonas for *T. congolense*, Duttonella for *T. vivax*, and Trypanozoon for *T. brucei* ssp.

Tsetse-transmitted trypanosomosis is a disease complex caused by several of these species, mainly transmitted cyclically by the genus *Glossina* (tsetse flies), but also mechanically by biting flies. Tsetse infest 10 million square kilometres and affect 37 countries, mostly in Africa, where the disease is known as 'nagana'. The disease infects various species of mammals but, from an economic point of view, tsetse-transmitted trypanosomosis is particularly important in cattle (also referred as tsetse-fly disease in southern Africa). It is mainly caused by *Trypanosoma congolense, T. vivax* and, to a lesser extent, *T. brucei brucei. Trypanosoma uniforme*, and *T. simiae* are other, less common tsetse-transmitted species. *Trypanosoma vivax* is also transmitted mechanically by biting flies, among which tabanids and stomoxes are presumed to be the most important, as exemplified by its presence in South and Central America, but also as observed in some areas of Africa free or cleared of tsetse (Ethiopia, Chad, etc.). Tsetse-transmitted trypanosomosis can affect camels and is a natural barrier preventing the introduction of this mammalian species into the southern Sahel region of West Africa. Horses are also highly sensitive. Very rare human cases have been observed caused by animal *Trypanosoma* species. However, tsetse-transmitted trypanosomosis also affects humans, causing sleeping sickness, through infection with either *T. brucei gambiense* or *T. brucei rhodesiense*. A large range of wild and domestic animals can act as reservoirs of these human parasites; particular care must be taken for people handling biological material that can contain infective human parasites, for example in livestock or wild fauna.

Clinical signs of tsetse-transmitted trypanosomosis may include intermittent fever, oedema, abortion, decreased fertility and emaciation. Anaemia usually develops in affected animals and is followed by loss of body condition, reduced productivity and often mortality. Post-mortem signs may include emaciation, enlarged lymph nodes, enlarged liver and spleen, excessive fluid in the body cavities, and petechial haemorrhages. In animals that died during the chronic phase of the disease, the lymphoid organs are usually no longer enlarged and severe myocarditis is a common finding. Neither clinical nor post-mortem signs of tsetse-transmitted trypanosomosis are pathognomonic. Therefore, diagnosis must rely on direct techniques that confirm the presence of trypanosomes either by microscopic visualisation or by indirect serological techniques or by polymerase chain reaction (PCR). Clinically, trypanosomosis can be confused with babesiosis, anaplasmosis, theileriosis, hemonchosis and even ehrlichiosis, rabies or plant intoxications. Differential diagnosis is oriented by clinical observations, evolution, epidemiological context, but it is essentially based on laboratory diagnosis.

B. DIAGNOSTIC TECHNIQUES

A variety of diagnostic tests are available (Toure, 1976) and researchers are still trying to improve existing tests and to develop new ones. Current diagnostic tests vary in their sensitivity and specificity, the ease with which they can be applied and their cost (Paris *et al*., 1982). The choice of a particular test will be guided by economic principles and the availability of expertise, but especially by the diagnostic requirement. For example, different degrees of sensitivity and specificity are applied to the confirmation of the infection in an individual animal as compared to the detection of infection at a herd level. Similarly, the diagnostic test(s) to establish the parasitological prevalence of trypanosomosis are different from those required to establish the presence or absence of the disease in an area. Reliable diagnosis may be achieved by combining appropriate diagnostic tests. Reliable interpretation of results from diagnostic tests will depend on test validity as well as on proper sample selection/collection, the sample size, and the way the diagnostic tests are conducted (see the Table 1).

Table 1. Test methods available for the diagnosis of tsetse-transmitted trypanosomosis and their purpose

		Type of area					
		Non-infected area			**Enzootic area**		**Both areas**
Type of diagnosis	**Purpose\ Method**	Confirmation & identification of suspected case	Population freedom from infection	Efficiency of eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Specific characteristics or interest
Agent id.	Thin stained blood smear	+++	=	=	++	±	Parasite morphology
	DNA detection/ PCR	+++	+++	+++	+++	+++	Sensitive & specific molecular identification

		Type of area					
		Non-infected area			Enzootic area		Both areas
Type of diagnosis	Purpose\ Method	Confirmation & identification of suspected case	Population freedom from infection	Efficiency of eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Specific characteristics or interest
Detection of active infection	Wet blood film	++	=	=	++	±	Fast follow-up (experimental) infection
	Thick stained blood film	=	=	=	±	±	Cheap technique
	Haematocrit centrifuge technique (HCT, Woo)	+++	+++	+++	+++	+++	Active infection & haematocrit value
	~~Dark ground method~~ Buffy coat technique (Murray)	++~~+~~	=	~~+~~–	++~~+~~	++	Active infection & haematocrit value
	Anion exchange columns	+++	=	+++	=	=	Small individual test or large parasite production
Parasite propa-gation	Rodent inoculation	+++	±	+++	=	=	Sensitive parasite isolation or production
	In-vitro culture	=	=	=	=	=	Parasite production
Serological diagnosis	IFAT	+++	++	=	=	++	Small-scale studies
	ELISA	+++	+++	+++	=	+++	Population studies

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose.
Although not all of the tests listed as category +++ or ++ have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.
Agent id. = agent identification; PCR = polymerase chain reaction; IFAT = indirect fluorescent antibody test; ELISA = enzyme-linked immunosorbent assay.

1. Identification of the agent

Parasite detection techniques are highly specific, but their sensitivity is relatively low (i.e. the proportion of false-negative results recorded is high). Sensitivity is especially low when results are considered at the individual animal level rather than the herd level. Sensitivity is highly variable during the course of the infection: (i) in the early phase, the sensitivity is high as parasites are actively multiplying in the blood in the absence of immunological control; (ii) during the chronic phase the sensitivity is low as, due to the immune response of the host, parasites are scanty and rarely seen in the blood; (iii) finally the sensitivity is almost nil in healthy carriers, where parasites are never seen. At the population level these variations mean that parasite detection techniques are highly sensitive during epizootic outbreaks (when most of the animals are in the early stages of infection), and are of low or very low sensitivity in enzootic areas (most of the animals are in the chronic stages of infection), especially during subclinical phases of the infection (healthy carriers). Due to this low sensitivity, the apparent parasitological prevalence of trypanosomosis is a little or much lower than the true parasitological prevalence. The low diagnostic sensitivity also makes it difficult to detect trypanosomosis when present at low parasitological

prevalence and it is impossible to establish the absence of the disease with a high degree of confidence. Moreover, in areas where trypanocidal drugs are used extensively, parasites may not be detected.

Several parasite detection techniques are available, each with varying sensitivity. The choice will depend on the laboratory facilities available and the aim of the diagnosis.

Direct examination techniques

The simplest techniques are examination of wet, thick or thin films of fresh blood, usually obtained from the ear vein, jugular vein or the tail. Amongst the direct examination techniques, stained thin blood films are generally regarded as more specific but less sensitive than the other two. The actual specificity and sensitivity of these techniques is directly dependent on the volume of blood actually examined and the skill and experience of the microscopist.

a) Wet blood films

These are made by placing a droplet of blood (about 2 µl) on a clean microscope slide and covering with a cover-slip (22 × 22 mm). The blood is examined microscopically at ×400 total magnification with condenser aperture, phase-contrast or interference contrast. Approximately 50–100 fields are examined. Trypanosomes can be recognised by their movement among the red blood cells (RBCs).

The method is simple, inexpensive and gives immediate results. Depending on the trypanosome size and movements a presumptive diagnosis can be made of the trypanosome species. Final confirmation of the species is made by the examination of the stained preparation.

The diagnostic sensitivity of the method is generally low but depends on the examiner's experience and the level of parasitaemia. Sensitivity can be improved significantly by lysing the RBCs before examination using a haemolytic agent such as sodium dodecyl sulphate (SDS).

b) Thick blood films

These are made by placing a drop of blood (5–10 µl) on a clean microscope slide and spreading it over an area of approximately 2 cm in diameter, using the corner of another slide. The thickness of the resultant film should be such that, when dry, the figures on a wristwatch dial can just be read through it. The film is dried thoroughly by rapidly waving in the air and, without fixation, is dehaemoglobinised by immersion in distilled water for a few seconds and dried before staining. A dry smear should be kept dry and protected from dust, heat, flies and other insects. It is stained for 30 minutes with 4% diluted Giemsa stain in phosphate buffered saline, pH 7.2. Staining time and stain dilution may vary with stain and individual technique. Therefore, it is important to start with the manufacturer's directions and to vary staining time and stain concentration to obtain the optimal result. The stained smear is then washed with buffered water and examined at ×500 to ×1000 total magnification.

The method is simple and relatively inexpensive, but results are delayed because of the staining process; however commercial kits are available for quick staining. Trypanosomes are easily recognised by their general morphology, but may be damaged during the staining process. This may make it difficult to identify the species.

c) Thin blood smear films

Thin blood smears are made by placing a small drop of blood (about 5 µl), for example from a microhaematocrit capillary tube, on a clean microscope slide approximately 20 mm from one end (allowing for space to apply the thick smear) and spreading with the edge of another slide. This slide is placed at an angle of approximately 30° to the first slide and drawn back to make contact with the blood droplet. The blood is allowed to run along the edge of the spreader, which is then pushed to the other end of the slide in a fairly rapid but smooth motion. If the correct amount of blood is used, the slide should be covered with a film of blood with no surplus before the end of the slide is reached, and the smear should take the shape of a bullet. Ideally, thin films should be prepared so that the RBCs are fairly close to each other but not overlapping. The slide is dried quickly by waving in the air and protected from dust, flies and other insects. The slide is fixed for 3 minutes in methanol, and stained as for thick blood smears. After staining, the slide is washed gently under tap water and allowed to dry. A variation of this method is to fix in methanol for 2 minutes, apply May–Grünwald stain for 2 minutes, then add an equal volume of buffered water, pH 7.2, leave for a further 8 minutes and drain off. Approximately 50–100 fields of the stained thin smear are examined, with a ×50 or ×100 oil-immersion objective lens, before the specimen is considered to be negative. Even after a trypanosome has been detected, approximately 20 extra fields are investigated to determine if more than one species is present. The sharp extremity of the smear must be extensively explored as, because of their capillary properties, trypanosomes may be concentrated at this place (especially true for large species like *T. brucei* and *T. vivax*).

The technique described above can also be used for biopsy samples of lymph obtained from punctured lymph nodes.

Usually, both a thin and thick smear is made from the same sample. Thick smears contain more blood than thin smears and, hence, have a higher diagnostic sensitivity. Thin smears on the other hand allow *Trypanosoma* species identification. Trypanosome species can be identified by the following morphological characteristics:

Trypanosoma vivax: 20–27 µm long, undulating membrane is medium or not obvious, free flagellum present at the anterior end, posterior end rounded, kinetoplast large and terminal.

Trypanosoma brucei is a polymorphic trypanosome species. Two distinctly different forms can be distinguished, i.e. a long slender form and a short stumpy form. Often, intermediate forms, possessing characteristics of both the slender and stumpy forms, are observed. The cytoplasm often contains basophilic granules in stained specimens.

- *Trypanosoma brucei* (long slender form): 17–30 µm long and about 2.8 µm wide, undulating membrane is conspicuous, free flagellum present at the anterior end, posterior end pointed, kinetoplast small and subterminal.

- *Trypanosoma brucei* (short stumpy form): 17–22 µm long and about 3.5 µm wide, undulating membrane is conspicuous, free flagellum absent, posterior end pointed, kinetoplast small and subterminal.

- *Trypanosoma congolense*: 8–25 µm (small species), undulating membrane not obvious, free flagellum absent, posterior end rounded, kinetoplast is medium sized and terminal, often laterally positioned. Although *T. congolense* is considered to be monomorphous, a degree of morphological variation is sometimes observed. A number of morphotypes have been described so far; from the slender to the stumpiest: hyperleptomorph (rodhaini-form, very long and slender, with a free flagellum), leptomorph (simiae-form, slender, with a free flagellum), isomorph (congolense-form, short, without a free flagellum), pachymorph (montgomeryi-form, short and stout; 0.25<WLr<0.34, without a free flagellum), and hyperpachymorph ('hyper-montgomeryi-form', short and very stout; 0.35<WLr<0.7, without a free flagellum) (Desquesnes *et al.,* 2012). Additionnally, sphaeromorph and rosettes have also been described. Within *T. congolense,* different types or subgroups exist (savannah, forest, kilifi or Kenya coast ~~tsavo~~) that have a different pathogenicity (Bengaly *et al*., 2002); also there is a large variation in pathogenicity within the savannah subgroup. These types can only be distinguished using PCR.

Trypanosoma theileri: (large species), typically 60–70 µm but individual organisms can range from 19 to 120 µm ~~(Levine, 1985; Matthews, 1979)~~, undulating membrane is conspicuous, long free flagellum present, posterior end pointed and rigid, kinetoplast is large and positioned near the nucleus and in a marginal position. *Trypanosoma theileri* is normally nonpathogenic, but its presence can confuse the parasitological diagnosis. In Western Europe, *T. theileri* is the only trypanosome species occurring in cattle.

Parasite concentration techniques

The probability of detecting trypanosomes in a sample from an infected animal depends largely on the amount of blood examined and the level of parasitaemia. The amount of blood examined with direct examination techniques is low and parasites are often very scanty in the blood of an infected animal. Both of these factors contribute to the low sensitivity of direct examination techniques. Sensitivity can be improved by increasing the volume of blood to be examined and by concentrating the trypanosomes.

a) Microhaematocrit centrifugation technique (Woo method)

The microhaematocrit centrifugation technique, or the Woo method (1970), is widely used for the diagnosis of animal trypanosomosis. It is based on the separation of the different components of the blood sample depending on their specific gravity. The method is as follows:

i) Fresh, usually ear vein blood (about 70 µl) is collected into heparinised capillary tubes (75 × 1.5 mm).

ii) One end of the capillary tube is sealed with cristaseal or by heating, ensuring that the column of blood is not charred by the flame.

iii) The sealed capillary tubes are placed in a microhaematocrit centrifuge with the sealed ends pointing towards the outside. To ensure good balance, the tubes are loaded symmetrically.

iv) The rotary cover is screwed on and the centrifuge lid is closed.

v) The capillary tubes are centrifuged at 9000 ***g*** for 5 minutes.

vi) A tube carrier is made from a slide on which two pieces of glass 25 × 10 × 1.2 mm have been fixed, 1.5 mm apart, to form a groove.

vii) The tube is placed in the groove, a cover-slip is placed on top and the interface is flooded with water. Alternatively, examination can be done without flooding the interface with water, but in such case, the light condenser must be placed in such a way that cells become refringent.

viii) The plasma/white blood cell interface (buffy coat) is examined by slowly rotating the tube. Trypanosome movement can first be detected using the ×10 objective lens with reduced condenser aperture; the trypanosomes can be seen more clearly using the ×40 objective lens preferably with a long working distance to allow adequate depth of focus through the capillary tube.

The microhaematocrit centrifugation technique is more sensitive than the direct examination techniques (Kratzer & Ondiek, 1989). In the case of *T. vivax* infections, the sensitivity of the Woo methods approaches 100% when the parasitaemia is >700 trypanosomes/ml blood. Sensitivity decreases to 50% when parasitaemia varies between 60 and 300 trypanosomes/ml blood. Trypanosomes become very difficult to detect when the parasitaemia is lower than 60 trypanosomes/ml blood (~~Desquesnes & Tresse, 1996~~ Desquesnes, 2004). Identification of trypanosome species is difficult. As the specific gravity of *T. congolense* is similar to that of RBCs, parasites are often found below the buffy coat in the RBC layer. To improve the separation of RBCs and parasites, and increase the sensitivity for *T. congolense*, the specific gravity of RBCs can be increased by the addition of glycerol.

A modification of the Woo method is the quantitative buffy coat method (QBC) (Bailey & Smith, 1992). The method has been used for the diagnosis of *T. b. gambiense* infections; it is generally too expensive for the routine large-scale use in animal trypanosomosis surveys.

b) Dark-ground/phase-contrast buffy coat technique (Murray method)

The buffy coat technique or Murray method (Murray *et al*., 1977) represents an improved technique for the detection of trypanosomes and is widely used. It is carried out following steps (i) to (v) above, after which the capillary tube is cut, with a diamond tipped pencil, ~~1~~ 0.5 mm below the buffy coat, to include the top layer of RBCs. The buffy coat and the uppermost layer RBCs are extruded on to a clean microscope slide (check that the buffy coat is not sticking to the capillary tube; it should be visible on the slide before covering it with a cover-slip [22 × 22 mm]). Approximately 200 fields of the preparation are examined for the presence of motile trypanosomes with a dark-ground or a phase-contrast microscope with a ×40 objective lens. Trypanosome species can be identified by reference to the following criteria:

Trypanosoma vivax: Large, extremely active, traverses the whole field very quickly, pausing occasionally.

Trypanosoma brucei: Various sizes, rapid movement in confined areas; undulating membrane traps the light into 'pockets' moving along the body.

Trypanosoma congolense: Small, sluggish, adheres to RBCs by anterior end.

Trypanosoma theileri: More than twice the size of pathogenic trypanosomes, tends to rotate; the posterior end is clearly visible, very long, sharp and rigid.

As with the microhaematocrit centrifugation technique, the buffy coat technique is more sensitive than direct examination techniques. The sensitivity of the buffy coat method can be improved by using the buffy coat double-centrifugation technique (Kratzer & Ondiek, 1989). A total amount of 1500–2000 µl of blood is centrifuged, after which the buffy coat is aspired into a microhaematocrit capillary tube and centrifuged again. The buffy coat is examined. However, collection of the buffy coat after the initial centrifugation is a delicate step and results may vary from one technician to another.

Compared with the microhaematocrit centrifugation technique, the buffy coat technique has the added advantage that preparations can be fixed and stained for more accurate identification of species and for retention as a permanent record.

Both the microhaematocrit centrifugation and buffy coat techniques give direct results and can be used for screening large numbers of animals. They require specialised equipment and an electricity supply making the test more expensive compared with the examination of the wet blood film. However, this is compensated for by increased sensitivity. Both parasite concentration techniques rely on the detection of motile, live, trypanosomes. Because trypanosomes can lose their vigour and die rather quickly once the blood sample is drawn, samples collected in capillary tubes should be cooled immediately and not be allowed to overheat in the microhaematocrit centrifuge or on the microscope stage. Samples should be examined as soon as possible after collection, preferably within a couple of hours.

The microhaematocrit centrifugation and buffy coat techniques are particularly useful in that the packed cell volume (PCV) can be assessed at the same time. To determine the PCV after centrifugation, the microhaematocrit capillary tube (containing ear vein or jugular vein blood) is placed in a haematocrit reader. The length of the packed RBC column is expressed as a percentage of the total volume of blood. Measuring the PCV is useful for determining the degree of anaemia. Anaemia can be caused by factors other than tsetse-transmitted trypanosomosis. It remains, however, one of the most important indicators of trypanosomosis in cattle. As trypanosomosis is a herd problem, the PCV-profile of a herd is influenced by the number of trypanosome-infected animals and can be used to indicate differences in disease challenge. The average PCV is also influenced by the age and level of genetic susceptibility of cattle.

c) Anion exchange

The miniature anion-exchange chromatography technique (m-AECT) is widely used for the diagnosis of human sleeping sickness caused by *T. b. gambiense* (Lumsden *et al*., 1979). Blood is passed through a diethyl amino-ethyl (DEAE)-cellulose column equilibrated with a phosphate buffered saline (PBS) solution of an ionic strength suited to the blood of the animal species under examination. As the RBCs are more negatively charged than the trypanosomes, they are held in the column and the trypanosomes pass through with the eluate, which is collected, centrifuged to concentrate the trypanosomes and examined under the microscope.

Large volumes of blood can be examined from each animal and, therefore, the method has high sensitivity. However, the technique is cumbersome and is not suitable for the examination of a large number of animals because it is very expensive and time consuming.

d) *In-vitro* cultivation

A procedure for the *in-vitro* cultivation of *T. brucei* has been described, but success has been irregular over many years. Moreover, the method needs sophisticated equipment, produces results after a considerable delay and is certainly not suitable for large-scale use. A kit for *in-vitro* isolation of trypanosomes has proven to be promising in isolating and amplifying all species of *T. brucei* in humans, domestic and game animals (Truc *et al*., 1992). The test's value in isolating *T. congolense* and *T. vivax* is still unknown. As it is based on the cultivation of procyclic forms of trypanosomes, species differentiation is not possible ~~(Komoin-Oka *et al*., 1994)~~; however a recent method has been described for a complete *in-vitro* life-cycle of *T. congolense* (Coustou *et al.,* 2010). It should be noted that cultivation is a highly efficient and sensitive method for the detection of tabanid-transmitted *T. theileri*, the prevalence of which is often found to be close to 100% using this technique. In the case of mixed infections, *T. theileri* easily overgrows *T. b. brucei* (Verloo *et al*., 2000).

Animal inoculation

~~The subinoculation of blood into rodents, usually mice or rats, is particularly useful in revealing subpatent infections. The laboratory animals are injected intraperitoneally with 0.2–5 ml (depending on the size of the rodent) of freshly collected blood. Artificial immunosuppression of recipient animals by irradiation or drug treatment will greatly increase the chances of isolating the parasite. They are bled three times a week for at least 2 months. Collected blood is examined using the wet film method.~~

~~Animal inoculation is more sensitive than direct examination of the wet blood film. Also the method is not practical; it is expensive and diagnosis is not immediate. The method is highly sensitive in detecting *T. b. brucei* infections (it is a technique of choice for detection of the non-tsetse-transmitted animal trypanosome: *T. evansi*). However, some *T. congolense* strains are not easily transmitted and *T. vivax* rarely infects laboratory rodents. Also animal inoculation should be avoided as it raises serious animal welfare concerns.~~

Rodent inoculation is expensive, diagnosis is not immediate, and the method should be avoided as much as possible as it raises serious animal welfare concerns. However, the inoculation of blood into rodents, usually mice or rats, is more sensitive than the Haematocrit centrifuge technique and sometimes PCR as well, thus it is particularly useful in revealing subpatent infections, which may be especially important in non-endemic areas.

The laboratory animals are injected intraperitoneally with 0.1–0.5 ml (depending on the size of the rodent) of freshly collected blood. Artificial immunosuppression of recipient animals by irradiation or drug treatment (cyclophosphamide 200 mg/kg) will greatly increase the chances of isolating the parasite. A drop of blood is collected from the tip of the rodent's tail three times a week. The blood is examined using the wet film method. If an infection occurs, it generally shows after 3–10 days, however the rodents must be followed for at least 1 month.

In cases of suspected trypanosome infection, the success rate of this method depends on the *Trypanosoma* species involved: it is highly sensitive for detection of *Trypanozoon* infections, of medium sensitivity for *T. congolense* strains, and generally poor but in rare cases effective for *T. vivax*.

The modern use of rodent inoculation should therefore be restricted to (i) massive parasitic antigen-production for serological diagnosis, and (ii) attempts at parasite demonstration and isolation when a trypanosome infection is suspected in animals living in or travelling towards a non-endemic area.

Test to detect trypanosomal antigen

Antigen-detection enzyme-linked immunosorbent assays (ELISA) for trypanosomosis have been described (Nantulya & Lindquist, 1989). Field evaluations of the tests have given inconsistent results (~~Desquesnes, 1997a;~~ International Atomic Energy Agency [IAEA], 1993). Additional works have been done under controlled conditions, which led to the conclusion that the sensitivity and specificity of these tests are not suitable for the diagnosis of trypanosomosis (Desquesnes, 1996; ~~1997;~~ 2004; Eisler *et al*., 1998).

DNA amplification tests

A PCR method has been developed as a tool for the diagnosis of infections with African trypanosomes in humans and animals, as well as tsetse flies. Specific repetitive nuclear DNA sequences can be amplified for *T. vivax* and three types of *T. congolense* (~~Desquesnes, 1997a;~~ Desquesnes & Davila, 2002; Masiga *et al*., 1992)~~; however current primers for *T. vivax* seem to not be able to amplify some genotypes within this species~~. A common primer set is available for detection of the three *T. brucei* subspecies. The primer sets available for different trypanosome subgenus, species and types are referred to as follows: Trypanozoon subgenus – TBR1 and TBR2; *T. congolense* (savannah type) – TCN1 and TCN2; *T. congolense* (forest type) – TCF1 and TCF2; *T. congolense* (Kenya Coast type) – TCK1 and TCK2; and *T. vivax* – TVW1 and TVW2. Due to the multiplicity of these taxon-specific primers in tsetse flies or cattle, a full trypanosome species identification requires that five PCR test be carried out per sample, which considerably increases the cost of diagnosis. Recently PCR restriction fragment length polymorphism (RFLP) assays and ITS1 of ribosomal DNA amplification have been developed that allow the identification of all *Trypanosoma* species as single or mixed infections using one single test (Delespaux *et al*., 2003; Desquesnes & Davila, 2002; Desquesnes *et al*., 2001; Geysen *et al*., 2003~~ 1997~~); however these tests are not yet suitable for routine diagnosis. Loop-mediated isothermal amplification is also under development for trypanosome diagnosis (Kuboki *et al*., 2003).

Standard monovalent PCR amplifications are carried out in a reaction mixture containing Tris/HCl, $MgCl_2$, KCl, each of the four deoxyribonucleotide triphosphates, primers, DNA template and Taq DNA polymerase. Samples are incubated during several cycles at varying temperatures. The PCR products are electrophoresed through agarose. Gels are stained with ethidium bromide and visualised under UV light for the presence of specific weight products.

The procedure is extremely sensitive, but false-positive results may occur as a result of contamination of samples with other DNA. The test requires specialised equipment and highly trained personnel, so it is not suitable for use in many laboratories. False-negative results may occur when the parasitaemia is very low (< 1 trypanosome/ml of blood), which occurs frequently in chronic infections; they may also occur when the specificity of the primers is too high, so that not all isolates of a particular trypanosome species are recognised. Sample collection has been simplified by adapting the test using blood or buffy coats spotted on to filter paper (Geysen *et al*., 2003~~ 1997~~; Katakura *et al*., 1997). A large number of samples can be processed at one time, making it potentially suitable for large-scale surveys. However, at the moment, the cost of PCR analyses is prohibitive for the routine use of the test.

2. Serological tests

Several antibody detection techniques have been developed to detect trypanosomal antibodies for the diagnosis of animal trypanosomosis, with variable sensitivity and specificity. The methods of choice are the indirect fluorescent antibody test (IFAT) (Katende *et al*., 1987) and the trypanosomal antibody-detection ELISA (Hopkins *et al*., 1998; Luckins, 1977). The identification of major antigens of trypanosomes, and their production as recombinant molecules or synthetic peptides, should hopefully lead to the development of new tests based on the use of defined molecules. Thus, in the future, it may be possible to improve the specificity of serological tests to allow the detection of species-specific antibodies, and to reach a high level of standardisation that is currently not achieved by the use of total parasite extracts.

a) Indirect fluorescent antibody test

The ~~original method for this test (Wilson, 1969) has been replaced by a new~~ technique for the preparation of trypanosomal antigens (Katende *et al*., 1987) involves fixation of live trypanosomes using a mixture of 80% cold acetone and 0.25% formalin in normal saline.

Test procedure

i) Prepare thin smears from heavily parasitaemic blood or from a trypanosome suspension. Air-dry and fix in acetone for 5 minutes.

ii) Mark circles of 5 mm diameter on glass slides using nail varnish.

iii) Using a pipette, place a test serum, diluted 1/40, in each circle, ensuring that the area in each circle is completely covered.

iv) Incubate the antigen/test serum preparation at 37°C for 30 minutes in a humid chamber.

v) Wash the preparation three times in PBS for 5 minutes each time at 4°C, with gentle agitation. Air-dry the slides.

vi) Apply conjugate: rabbit or goat anti-bovine IgG (for tests on bovine sera) conjugated to fluorescein isothiocyanate.

vii) Incubate and wash as above. Rinse in distilled water. Air-dry the slides.

viii) Mount the slides in PBS or buffered glycerol and examine for fluorescence.

b) Antibody-detection enzyme-linked immunosorbent assay

The original antibody ELISA (Luckins, 1977) has recently been further developed for use in large-scale surveys of bovine trypanosomosis (Desquesnes, 1997~~b~~; Hopkins *et al*., 1998). Recommendations have been made that allow antigen production and standardisation of the test on a local basis (Desquesnes, 1997; 2004; Greiner *et al*., 1997; Wright *et al*., 1993).

The standard antigen for trypanosomosis antibody tests is derived from bloodstream-form trypanosomes. Trypanosomes are purified by DEAE anion-exchange chromatography of parasites from whole blood of infected rats (Lanham & Godfrey 1970). Antigens are prepared as a soluble fraction with lysis (with the addition of anti-enzyme) using seven freeze–thaw cycles and centrifugation at 10,000 ***g*** for ~~30~~ 10 minutes. Antigens obtained from *in-vitro* propagated procyclic trypanosome forms can also be used (Greiner *et al*., 1997). Soluble antigens must be added with a protease inhibitor cocktail and be stored at –80°C or –20°C for long and short periods, respectively, but they may also be lyophilised for conservation at room temperature. ELISAs using *T. congolense* or *T. vivax* precoated microtitre plates have been developed that have the advantage that a standardised denatured antigen is used that can be stored for long periods at room temperature (Rebeski *et al*., 2000).

Both the IFAT and antibody-detection ELISA have been adapted for the analysis of blood samples collected on filter paper. Blood contained in one heparinised microhaematocrit centrifuge capillary tube is extruded on to a filter paper (Whatman® No. 4). Samples are air-dried out of direct sunlight and placed in a plastic bag with self-indicating silica gel desiccant. The bag is sealed and should be kept as cool as possible until specimens are refrigerated or frozen.

Each ELISA-microplate is run with strong positive, weak positive and negative reference sera, which are required to comply with pre-set values for quality assurance. The absorbance of each ELISA-sample tested is expressed as a percentage (percentage positivity: PP) of the strong positive reference standard (Wright *et al*., 1993), or the positive and negative reference standards (Desquesnes, 1997~~b~~); results are, therefore, quantifiable. The cut-off value is determined using known positive and negative field or experimental samples (Desquesnes, 1997~~b~~; 2004).

Both antibody-detection tests have high sensitivity and genus specificity. Their species specificity is generally low, but may be improved by using a standardised set of the three species-specific tests (Desquesnes, 2004). They detect immune responses to current and past infections and can, therefore, only provide a presumptive diagnosis of active infection. However, persistence of antibodies after a curative treatment or a self-cure is estimated to be on the average of 3–4 months in young and adult cattle infections (Desquesnes, 2004); although it might take up to 13 months before all antibodies have disappeared in some animals (Van den Bossche *et al*., 2000) consequently, proper sampling and knowledge of trypanocidal use will give more acute information.

Immunodiagnosis needs expensive, sophisticated equipment and expertise, which is not always available. It has to be performed in specialised laboratories and there is a substantial delay between the actual sampling and the availability of the results. Nevertheless, the antibody ELISA lends itself to a high degree of automation and standardisation. Sample collection and storage is made easy through the use of filter papers. All of these factors make the antibody ELISA a very useful test for large-scale surveys to determine the distribution of tsetse-transmitted trypanosomosis.

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

No vaccines are in use at the present time. ~~The only requirement for diagnostic biologicals in livestock is to grow parasites in rodents to avoid the presence of livestock components in IFAT and ELISA biologics.~~

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* *

NB: There is an OIE Reference Laboratory for Trypanosomosis
(see Table in Part 4 of this *Terrestrial Manual* or consult the OIE web site for the most up-to-date list:
http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/ http://www.oie.int/).
Please contact the OIE Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for Trypanosomosis (tsetse-transmitted)

CHAPTER 2.5.3.

DOURINE

SUMMARY

Dourine is a chronic or acute contagious disease of breeding equids ~~solipeds~~ that is transmitted directly from animal to animal during coitus. The causal organism is Trypanosoma (Trypanozoon) equiperdum (*Doflein, 1901*).

Dourine is the only trypanosomosis that is not transmitted by an invertebrate vector. Trypanosoma equiperdum *differs from other trypanosomes in that it is primarily a tissue parasite that is rarely detected in ~~invades~~ the blood. There is no known natural reservoir of the parasite other than infected equids. It is present in the genital secretions of both infected males and females. The incubation period, severity, and duration of the disease vary considerably; it is often fatal, however it was claimed that ~~but~~ spontaneous recoveries do occur and ~~but~~ latent carriers do exist as well. Subclinical infections can occur. Donkeys and mules are more resistant than horses and may remain unapparent carriers. Infection is not always transmitted by an infected animal at every copulation. Although adaptation to other hosts is not always possible, laboratory rodents, such as rabbits, rats and mice, ~~Rats~~ can be infected experimentally and can be used to maintain strains of the parasite indefinitely.* Trypanosoma equiperdum *strains are best stored in liquid nitrogen.*

The clinical signs are marked by periodic exacerbation and relapse, ending in death, sometimes after paraplegia or, possibly, recovery. Moderate fever, local oedema of the genitalia and mammary glands, cutaneous eruptions, incoordination, facial and lip paralysis, ocular lesions, anaemia, and emaciation may all be observed. Oedematous cutaneous plaques, 5–8 cm in diameter and 1 cm thick, are still considered as pathognomonic, although they were also found in equids infected with T. evansi *occasionally.*

Identification of the agent: *Definitive diagnosis depends on the recognition of clinical signs and identification of the parasite. As this is rarely possible, diagnosis is usually based on clinical signs, together with serological evidence from complement fixation* (*CF*) *tests.*

Serological tests: *Humoral antibodies are present in infected animals whether or not they display clinical signs. The CF test is used to confirm infection in clinical cases or in latent carriers. Non-infected animals, especially donkeys, often yield unclear results. The indirect fluorescent antibody test can be used to confirm infection or resolve inconclusive CF test results. Enzyme-linked immunosorbent assays are also used.*

Molecular tests: *Genetic markers which will allow differentiating* T. equiperdum *from* T. evansi *within the subgenus* Trypanozoon *are under investigation.*

Requirements for vaccines ~~and diagnostic biologicals~~: *There are no vaccines available for this parasite. The only effective control is through the slaughter of infected animals. Good hygiene is essential during assisted mating because infection may be transmitted through contaminated fomites.*

A. INTRODUCTION

Dourine is a chronic or acute contagious disease of equids ~~breeding solipeds~~ that is transmitted directly from animal to animal during coitus. The causal organism is *Trypanosoma equiperdum* (Doflein, 1901). Dourine is also known under other names: mal de coït, syphilis du cheval, el dourin, morbo coitale maligno, Beschälseuche, slapsiekte, sluchnaya bolyezni), and covering disease (Barner, 1963; Hoare, 1972). So far no human case has been reported.

Although the disease has been known since ancient times, its nature was established only in 1896 when Rouget discovered trypanosomes in an infected Algerian horses. Dourine is the only trypanosomosis that is not transmitted by an invertebrate vector. *Trypanosoma equiperdum* differs from other trypanosomes in that it is primarily a tissue parasite that is rarely detected in the blood. There is no known natural reservoir of the parasite other than infected equids.

Infection is transmitted during copulation, more commonly from stallion to mare, but also from mare to stallion, due to the presence of the parasite in the seminal fluid and mucous exudate of the penis and sheath of the infected male, and in the vaginal mucus of the infected female. Initially, parasites are found free on the surface of the mucosa or between the epithelial cells of a newly infected animal. Invasion of the tissues takes place, and oedematous patches appear in the genital tract. Parasites then may pass into the blood, where they are carried to other parts of the body. In typical cases, this metastatic invasion gives rise to characteristic cutaneous plaques.

The incubation period, severity and duration of the disease vary considerably. In South Africa, the disease is typically chronic, usually mild, and may persist from 6 months to 2 for several years (Henning, 1955). In other areas, such as Northern Africa and South America, the disease tends to be more acute, often lasting only 1–2 months or, exceptionally, 1 week. Although dourine is a fatal disease with an average mortality of 50% (especially in stallions), spontaneous recovery can occur. Subclinical infections are recognised. Donkeys and mules are more resistant than horses. In donkeys, the disease passes often unperceived whereas its semen and vaginal secretions contain infective trypanosomes. In 2011, an outbreak of dourine was reported in Italy, 16 years after the previous officially reported dourine case in that country (Pascucci *et al.*, 2013; Scacchia *et al.*, 2011).

As trypanosomes are not continually present in the genital tract throughout the course of the disease, transmission of the infection does not necessarily take place at every copulation involving an infected animal. Transmission of infection from mare to foal can occur via the mucosa, such as the conjunctiva. Mares' milk has been shown to be infectious. Trypanosomes were found in the mammary gland of a non-lactating mare (Parkin, 1948) and in skin samples after examination by immunohistochemistry (Pascucci *et al.*, 2013; Scacchia *et al.*, 2011). Animals other than equids can hardly be infected experimentally, e.g. rabbits, rats and mice. Thus the isolation of new strains is quite difficult. It has been reported that adaptation to rats is possible after isolation in rabbits by intratestis inoculation (Heisch *et al.*, 1970; Schneider & Buffard, 1900; Soldini, 1939). Rodent-adapted strains can be maintained indefinitely; infected rodent rat blood can be satisfactorily cryopreserved. Antigens for serological tests are commonly produced from infected laboratory rodents rats.

Dourine is marked by stages of exacerbation, tolerance or relapse, which vary in duration and which may occur once or several times before death or recovery. The clinical signs of this disease most frequently noted are: pyrexia, tumefaction and local oedema of the genitalia and mammary glands, oedematous cutaneous eruptions, knuckling of the joints, incoordination, facial and lips paralysis, ocular lesions, anaemia, and emaciation. A pathognomonic sign is the oedematous plaque consisting of an elevated lesion in the skin, up to 5–8 cm in diameter and 1 cm thick. The plaques usually appear over the ribs, although they may occur anywhere on the body, and usually persist for between 3 and 7 days. They are not a constant feature.

Generally, the oedema disappears and returns at irregular intervals. During each recess, an increasing extent of permanently thickened and indurated tissue can be seen. The vaginal mucosa may show raised and thickened semi-transparent patches. Folds of swollen membrane may protrude through the vulva. It is not uncommon to find oedema of the mammary glands and adjacent tissues. Depigmentation of the genital area, perineum, and udder may occur. In the stallion, the first clinical sign is a variable swelling involving the glans penis and prepuce. The oedema extends posteriorly to the scrotum, inguinal lymph nodes, and perineum, with an anterior extension along the inferior abdomen. In stallions of heavy breeds, the oedema may extend over the whole floor of the abdomen.

Pyrexia is intermittent; nervous signs include incoordination, mainly of the hind limbs, lips, nostrils, ears, and throat. Facial paralysis is usually unilateral. In fatal cases, the disease is usually slow and progressive, with increasing anaemia and emaciation, although the appetite remains good almost throughout.

At post-mortem examination, gelatinous exudates are present under the skin. In the stallion, the scrotum, sheath, and testicular tunica are thickened and infiltrated. In some cases the testes are embedded in a tough mass of sclerotic tissue and may be unrecognisable. In the mare, the vulva, vaginal mucosa, uterus, bladder, and mammary glands may be thickened with gelatinous infiltration. The lymph nodes, particularly in the abdominal cavity, are hypertrophied, softened and, in some cases, haemorrhagic. The spinal cord of animals with paraplegia is often soft, pulpy and discoloured, particularly in the lumbar and sacral regions.

B. DIAGNOSTIC TECHNIQUES

Table 1. *Test methods available and their purpose*

Method	Purpose			
	Population freedom from infection	Individual animal freedom from infection	Confirmation of clinical cases	Prevalence of infection – surveillance
Microscopy	–	+	+++	-
Complement fixation test	+	+++	+++	+++
IFAT	++	+	+	++
ELISA	++	+	+	++
PCR	–	+	++	+

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose.
Although not all of the tests listed as category +++ or ++ have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.
PCR = polymerase chain reaction; IFAT = indirect fluorescent antibody test;
ELISA = enzyme-linked immunosorbent assay.

1. Identification of the agent

a) Overview of parasitological methods

A definitive diagnosis depends on the recognition of the clinical signs and the demonstration of the parasite. This is rarely possible because: (a) although the clinical signs and gross lesions in the developed disease may be pathognomonic, they cannot always be identified with certainty, especially in the early stages or in latent cases; they can be confused with other conditions, such as coital exanthema (moreover, in some countries [e.g. in South America], *T. evansi* infections give rise to similar clinical signs; (b) the trypanosomes are only sparsely present and are extremely difficult to find, even in oedematous areas; and (c) the trypanosomes are only fleetingly present in the blood, and in small numbers that defy detection. ~~For unknown reasons~~ Illustrating the challenges to isolate *T. equiperdum*, no parasite strain ~~of~~ widely accepted to be *T. equiperdum* has been isolated in any country of the world since 1979 and most of the strains currently available in national veterinary diagnostic laboratories are related to *T. evansi* (Claes *et al.*, 2003; Lun *et al*., 1992).

Recently, new putative *T. equiperdum* strains have been isolated in Ethiopia (Dodola), in Italy (ICT 2011) and Venezuela (TeAp-N/D1), although these isolates still have to be further characterised (Hagos *et al*., 2010; Perrone *et al*. 2009; Pascucci *et al.,* 2013). In practice, diagnosis is based on clinical evidence supported by the mode of transmission, serology and histopathology. Recently, other approaches have been studied and reported on (Claes *et al.*, 2003).

In infected animals, trypanosomes are present, in low numbers only, in lymph and oedematous fluids of the external genitalia, in the vaginal mucus (Parkin, 1948) and exudates ~~fluid contents~~ of plaques and mammary gland exudates ~~milk~~ (Pascucci *et al*., 2013; Scacchia *et al*., 2011). They are usually undetectable in the blood, but may be found in the urethral or vaginal mucus collected from preputial or vaginal washings or scrapings 4–5 days after infection. Later, parasites may be found in the fluid contents of oedemas and plaques, especially shortly after their eruption. The skin of the area over the plaque should be washed, shaved and dried, and the fluid contents aspirated by syringe. Blood vessels should be avoided. The fresh aspirate is examined microscopically for motile trypanosomes. These are present for a few days only, so that lesions should be examined at intervals. As the parasite is rarely found in thick blood films, ~~but~~ the use of concentration techniques is recommended, such as capillary tube centrifugation, and mini anion exchange centrifugation technique (Büscher *et al*., 2009; Lanham & Godfrey, 1970; Woo 1970).

As dourine is the only trypanosome to affect horses in ~~temperate climates~~ areas free from Nagana or Surra diseases, the observation of trypanosomes in thick blood films is sufficient for a positive diagnosis. In countries where Nagana or Surra occur, it is difficult to distinguish *T. equiperdum* microscopically

(morphology, motility) from other members of the subgenus *Trypanozoon* (*T. evansi, T. brucei*). In particular, *T. equiperdum* and *T. evansi* cannot be differentiated on the basis of morphological criteria. Both are monomorphic, slender trypomastigotes with a free flagellum, although pleomorphic, stumpy ~~proteonuclear~~ forms are recognised. ~~However, in kinetopastic presence of maxi-circles in *T. equperdum* and the absence in *T. evansi* provides a possible differentiation (Wassal *et al.*, 1991).~~ Typical strains of the parasite range in length from 15.6 to 31.3 µm.

b) Detection of trypanosomal DNA and differential diagnosis

Kinetoplast DNA in the mitochondrion is the most remarkable characteristic in the Order Kinetoplastida. In field situations, akinetoplast strains of *T. evansi* (no kinetoplast was stained with Giemsa) were found in infected animals, but this situation was not observed in *T. equiperdum*. More relevant for distinction is ~~In kinetoplastic strains,~~ the presence of maxi-circles in *T. equiperdum* and the absence in *T. evansi,* which provides a possible differentiation between these two parasites (Li *et al.*, 2007). In addition, other publications suggest that absence of the VSG RoTat 1.2 could be a molecular marker to differentiate *T. equiperdum* from *T. evansi* (Claes *et al.*, 2003; 2004), however VSG RoTat 1.2 is absent from some *T. evansi* stains (Ngaira *et al.,* 2005). Although no *T. equiperdum*-specific polymerase chain reaction (PCR) method is available, subgenus *Trypanozoon*-specific PCR can be used for detection of *T. equiperdum* DNA (See Chapter 2.1.17 Surra). Recently, a highly sensitive real-time PCR for *Trypanozoon* subgenus was applied on tissues and fluid samples from a naturally dourine-infected horse, enabling the detection of low numbers of parasites (Pascucci *et al.*, 2013; Scacchia *et al.,* 2011).

PCR and other related DNA amplification methods have been used to examine exudates or tissue samples, taking into account their failure on blood samples after the initial phase of the infection (Calistri *et al.*, 2013).

2. Serological tests

Humoral antibodies are present in infected animals, whether they display clinical signs or not. The complement fixation (CF) test (UK Ministry of Agriculture, Fisheries and Food [MAFF], 1986) is used to confirm clinical evidence and to detect latent infections. Uninfected equids, particularly donkeys and mules, often give inconsistent or nonspecific reactions because of the anticomplementary effects of their sera. In the case of anticomplementary sera, the indirect fluorescent antibody (IFA) test is of advantage. There is no internationally adopted protocol. Cross-reactions are possible due to the presence in some countries of other trypanosomes, for example, *T. cruzi* and *T. evansi*. Enzyme-linked immunosorbent assays (ELISAs) are also used. *Trypanosoma equiperdum* is closely related to other Old World trypanosomes, including *T. brucei* and *T. evansi*. Members of this genus all share conserved cytoskeletal elements that provoke a strong and cross-reactive serological response. All diagnostic antigens and antisera ~~(monoclonal and polyclonal)~~ currently available for use in serodiagnostic testing contain these conserved elements or antibodies to them, and thus none of the serological procedures described below is specific for dourine. Therefore, the diagnosis of dourine must include history, clinical, and pathological findings as well as serology to establish the definite confirmed case of the disease (Calistri *et al.*, 2013). Significant improvements in dourine serodiagnosis will require development of more *T. equiperdum* ~~trypanosome~~-specific subunit antigens and antibodies to them.

a) Complement fixation test (the prescribed test for international trade)

Standard or microplate techniques may be used (Herr *et al.*, 1985). Guinea-pig serum (available commercially) is used as a source of complement. Other reagents are sheep red blood cells (RBCs) washed in veronal buffer, and rabbit haemolytic serum (i.e. rabbit anti-sheep RBC) (commercial) as well as known negative and positive control sera.

- **Antigen production**

Because of lack of solid serological or molecular markers to differentiate *T. equiperdum* from *T. evansi*, careful attention has to be paid to which *T. equiperdum* strain is used for the antigen preparation. According to recent progresses for differentiation of the two closely related species (Claes *et al.*, 2003, 2004), true *T. equiperdum* seems to be differentiated from *T. evansi* type A by absence of the VSG RoTat 1.2 gene. With this in mind, *T. equiperdum* OVI and BoTat 1.1 strains could be the suitable strains for antigen sources.

i) A specific-pathogen free rat is inoculated with *T. equiperdum* cryopreserved stock. ~~The rat must be free from *T. lewisi*, which could be achieved by injection with neoarsphenamine, but is better accomplished by using specific-pathogen free rats.~~ Adult rats receive 0.5–1.0 ml of rapidly thawed frozen stabilate intraperitoneally. At maximum parasitaemia, blood is collected into an anticoagulant, such as heparin, which will serve as a stock culture for the inoculation of additional rats.

ii) Twenty large rats are inoculated ~~intramuscularly or~~ intraperitoneally with 0.5–1.0 ml of this stock culture. All rats are to have a heavy infection concurrently. If necessary, the dose is adjusted and additional rats are inoculated to reach maximum parasitaemia at the desired time of 72–96 hours. Rats usually die within 3–5 days; prior to this, blood is taken from the tail for ~~thick smears~~ thin wet blood films and

examined microscopically. When parasitaemia is maximal, the rat is killed and blood is collected ~~in Alsevers or acid–citrate–dextrose (ACD) saline solution. If parasitaemia is not synchronous, blood can be collected and held in Alsevers or ACD saline at 4°C until blood has been collected from all the rats~~ for separation of the trypanosomes by one of the two protocols below: differential centrifugation or anion exchange chromatography.

iii) For differential centrifugation, infected rat blood is collected in Alsever's or acid–citrate–dextrose (ACD) saline solution. Although parasitemia is usually synchronous, if not, blood can be collected and held in Alsever's or ACD saline solution at 4°C until blood has been collected from all the rats. The blood is filtered through muslin gauze and centrifuged at 800 ***g*** for 4 minutes. The RBCs are mostly deposited while the trypanosomes remain in suspension.

iv) The supernatant fluid is transferred to a fresh tube; the upper layer of RBCs is mixed with trypanosomes to a second tube, and the next layer to a third. Alsever's or ACD saline solution is added to tubes 2 and 3 to prevent clotting of cells. All tubes are mixed and centrifuged at 1500 ***g*** for 5 minutes.

v) The supernatant fluid is discarded and the upper white layer of trypanosomes is transferred from all tubes into a clean tube. The next pink layer is transferred into a second tube, and the lower layer to a third tube.

vi) Physiological saline is added and mixed and the tubes are centrifuged again at 1500 ***g*** for 5 minutes to separate the trypanosomes. The washing step is repeated until all the trypanosomes are collected as a pure white mass. Ten rats should produce 3–5 g of antigen. ~~This purification procedure can also be carried out using a column of DEAE (diethylaminoethyl) cellulose in a solution of phosphate buffered saline (PBS) containing glucose, pH 8.0 (Lanham & Godfrey, 1970).~~ The concentrated trypanosomes are diluted with two volumes of veronal buffer and 5% polyvinylpyrrolidone as a cryopreservative. Before use in CF tests, the antigen must be dispersed to a fine suspension with a hand-held or motorised ground glass homogeniser chilled in ice (Watson, 1920). This antigen may be divided into aliquots, frozen and lyophilised.

vii) For anion exchange chromatography, blood is taken on heparin and loaded on a DEAE (diethylaminoethyl) cellulose gel equilibrated with a solution of phosphate buffered saline (PBS) containing glucose, pH 8.0 (Lanham & Godfrey, 1970). Blood cells are retained on the gel and the eluted trypanosomes are centrifuged at 1000–1500 ***g*** for 15 minutes. One volume of sedimented trypanosomes is resuspended in ice-cold phosphate buffer 0.01 M at pH 8.0 and thus lysed by hypotonic shock during 15 minutes. Thereafter, the suspension is centrifuged at 42,000 ***g*** for 1 hour and the supernatant is collected and filtered through a 0.22 µm filter. The cleared supernatant contains the hydro-soluble fraction of the trypanosomes. The protein content can be determined by UV spectrophotometry or similar method and this antigen preparation can be stored at –80°C in small volumes.

The antigen is standardised by titration against a 1/5 dilution of a standard low-titre antiserum.

Sera: Positive and negative sera should be inactivated at 58°C for 30 minutes before being used in the tests. Mule and donkey sera are normally inactivated at 62°C for 30 minutes. The USDA complement fixation protocol calls for inactivation of sera for 35 minutes (United States Department of Agriculture [USDA], 2006). Dilutions of sera that are positive in the screening test are titrated against two units of antigen. Test sera are screened at a dilution of 1/5. Sera showing more than 50% complement fixation at this dilution are usually deemed to be positive.

Anticomplementary sera: If the anticomplementary control shows only a trace, this may be ignored. For all other anticomplementary sera, the activity must be titrated. A duplicate series of dilutions is made and the sample is retested using *T. equiperdum* antigen in the first row and veronal buffer only in the second. The second row gives the titre of the anticomplementary reaction. Provided the first row shows an end-point that is at least three dilutions greater than the second, the anticomplementary effect may be ignored and the sample rated as positive. If the results are any closer, a fresh sample of serum must be requested. Dilution of the serum 1/2 and heat inactivation at 60–63°C for 30 minutes may result in reduction or removal of the anticomplementary effect.

Buffers and reagents: 0.15 M veronal buffered saline, pH 7.4, is used for diluting reagents and for washing sheep RBCs. Antigen is pretested by checkerboard titration, and two units are used in the test. Guinea-pig complement (C) is tested for its haemolytic activity, and diluted to provide two units for the test. Sheep RBCs in Alsever's or ACD saline solution are washed three times. A 3% solution is used for the haemolytic system. The USDA protocol calls for 2% solution in the microtitration procedure with confirmation in a tube test with 3% RBC (USDA, 2006). Titrated rabbit-anti-sheep RBCs – the rabbit haemolytic serum – is taken at double the concentration of its haemolytic titre (two units). All test sera, including positive and negative control sera, are inactivated at a 1/5 dilution before testing.

- **Primary dilutions**

~~i) 100 µl of test serum is diluted with 400 µl of veronal buffer (1/5).~~

i) ~~100 µl of both~~ Test, positive and negative control sera are diluted five-times with ~~400 µl of~~ veronal buffer ~~(1/5)~~.

ii) The solutions are incubated in a water bath at 58°C for 30 minutes to inactivate complement and destroy anticomplementary factors. Donkey and mule sera should be inactivated at 63°C for 30 minutes.

- **Screening test procedure**

i) 25 µl of inactivated test serum is placed in each of three wells.

ii) 25 µl of inactivated control serum is placed in each of three wells.

iii) 25 µl of *T. equiperdum* antigen diluted to contain two units is placed in the first well only for each serum.

iv) 25 µl of complement diluted to contain ~~two~~ five units is added to the first two wells only for each serum.

v) 25 µl veronal buffer, pH 7.4, is added to the second well for each serum (anticomplementary well).

vi) 50 µl veronal buffer, pH 7.4, is added to the third well for each serum (lysis activity well).

vii) The complement control is prepared. ~~(Optionally, an antigen control may be prepared.)~~

viii) The plate is shaken on a microshaker sufficiently to mix the reagents.

ix) The plate is incubated for 1 hour in a water bath, incubator or in a humid chamber at 37°C.

x) The haemolytic system is prepared. After the first 50 minutes of incubation, the sheep RBCs are sensitised by mixing equal volumes of rabbit haemolytic serum, diluted to contain two units per 50 µl, and a 3% suspension of washed RBCs; the solution is mixed well and incubated for 10 minutes at 37°C.

xi) After incubation, 50 µl of haemolytic system is added to each well.

xii) The plate is shaken on a microshaker sufficiently to mix the reagents.

xiii) The plate is incubated for 30 minutes at 37°C. To aid in reading the results, the plates can be centrifuged after incubation.

xiv) *Reading the results:* the plate is viewed from above with a light source beneath it. The fixation in every well is assessed by estimating the proportion of cells not lysed. The degree of fixation is expressed as 0, 1+, 2+, 3+, 4+ (0%, 25%, 50%, 75% or 100% cells not lysed). Reactions are interpreted as follows: 4+, 3+, 2+ = positive, 1+ = suspicious, trace = negative, complete haemolysis = negative.

xv) *End-point titration:* All sera with positive reactions at 1/5 are serially double diluted and tested according to the above procedure for end-point titration.

b) Indirect fluorescent antibody test

An IFA test for dourine can also be used (MAFF, 1986) as a confirmatory test or to resolve inconclusive results obtained by the CF test. The test is performed as follows:

Antigen: (For method, see preparation of CF test antigen in Section B.2.a) Blood is collected into heparinised vacutainers or into a solution of acid–citrate–dextrose from an animal in which the number of trypanosomes is still increasing (~~about ten~~ more than 10 parasites per 10×40 microscope field should be present).

i) The blood is centrifuged for 10 minutes at 800 ***g***.

ii) One to two volumes of PBS is added to the packed RBCs, the mixture is agitated, and smears are made that evenly cover the whole slide.

iii) The smears are air-dried and then wrapped in bundles of four, with paper separating each slide. The bundles of slides are wrapped in aluminium foil, sealed in an airtight container over silica gel, and stored at –20°C or ~~–76~~ 80°C.

iv) Slides stored at –20°C should retain their activity for about 1 year, at ~~–76~~ 80°C they should remain useable for longer.

Acid–citrate–dextrose solution: Use 15 ml per 100 ml of blood.

Conjugate: ~~Fluorescein~~ Fluorescence-labelled sheep anti-horse immunoglobulins.

- **Test procedure**

i) The antigen slides are allowed to reach room temperature in a desiccator. An alternative method is to remove slides directly from the freezer and fix them in acetone for 15 minutes.

ii) The slides are marked out.

iii) Separate spots of test sera diluted in PBS are applied, and the slides are incubated in a humid chamber ~~in a water bath~~ at ~~37°C~~ ambient temperature for 30 minutes.

iv) The slides are washed in PBS, pH 7.2, three times for 5 minutes each, and air-dried.

v) ~~Fluorescein~~ Fluorescence-labelled conjugate is added at the correct dilution. Individual batches of antigen and conjugate should be titrated against each other using control sera to optimise the conjugate dilution. The slides are incubated in a humid chamber ~~in a water bath~~ at ~~37°C~~ ambient temperature for 30 minutes.

vi) The slides are washed in PBS, three times for 5 minutes each, and air-dried. An alternative method, to reduce background fluorescence, is to counter-stain, using Evans Blue (0.01% in distilled water) for 1 minute, rinse in PBS and then air dry

vii) The slides are mounted in glycerol/PBS (50/50), ~~or~~ immersion oil (commercially available, non-fluorescing grade), or mounting reagent for fluorescent staining (commercially available).

viii) The slides are then examined under UV illumination. Incident light illumination is used with ~~barrier~~ a suitable filter ~~K 530 and exciter filter BG 12~~ set. Slides may be stored at 4°C for 4–5 days. Sera diluted at 1/80 and above showing strong fluorescence of the parasites are usually considered to be positive. Estimating the intensity of fluorescence demands experience on the part of the observer.

Standard positive and negative control sera should be included in each batch of tests, and due consideration should be given to the pattern of fluorescence in these controls when assessing the results of test sera.

c) Enzyme-linked immunosorbent assay (ELISA)

The ELISA has been developed and compared with other serological tests for dourine (Bundesinstitut für Gesundheitlichen Verbraucherschutz und Veterinärmedizin, 1995; Wassall *et al.,* 1991).

Carbonate buffer, pH 9.6, for antigen coating on to microtitre plates: Na_2CO_3 (1.59 g); $NaHCO_3$ (2.93 g); and distilled water (1 litre). Alternatively phosphate buffered saline (PBS) (KH_2PO_4 (0.2 g); $Na_2HPO_4 \times 12\ H_2O$ (2.94 g); NaCl (8.0 g); KCl (0.2 g in 1 litre distilled water)) can be used for preparation of the antigen solution.

Blocking buffer: Carbonate buffer + 3% fetal calf serum (FCS), or PBS + 1% (w/v) casein.

PBS, pH 7.4, with Tween 20 (*PBST*) *for washing:* PBS + 0.05% (v/v) ~~KH_2PO_4 (0.2 g); $Na_2HPO_4 \times 12\ H_2O$ (2.94 g); NaCl (8.0 g); KCl (0.2 g in 1 litre distilled water), and~~ Tween 20 ~~(0.5 ml)~~.

Sample and conjugate buffer: PBST + 6% FCS, or PBS + 1% (w/v) casein.

Citric phosphate buffer: Citric acid monohydrate (4.2 g in 200 ml distilled water); $Na_2HPO_4 \times 12\ H_2O$ (in 200 ml distilled water). Both components are mixed at equal volumes.

Substrate indicator system: ABTS (2,2'-Azino-bis-[3-ethylbenzothiazoline-6-sulphonic acid]) ~~(40 mg) is dissolved in citric phosphate buffer (100 ml), and stored at 4°C in the dark. Just before use, 100 µl of 1/40 H_2O_2 is added to 10 ml of ABTS~~ (commercially available).

Conjugate: Rabbit anti-horse IgG (H+L) PO or IgY anti-horse Ig-PO.

Antigen: For method, see preparation of CF test antigen in Section B.2.a.

~~*Antigen:* Lyophilised *T. equiperdum* antigen (0.5 ml) is reconstituted with coating buffer (5 ml), sonicated twice for 10 seconds each at 12 µm peak to peak, and centrifuged at 10,000 ***g*** for 4 minutes. The supernatant is further diluted to a pretested working dilution (e.g. 1/500).~~

- **Test procedure**

i) Wells in columns 2, 4, 6, etc., are charged with 100 ~~50~~ µl of antigen (2 µg/ml), columns 1, 3, 5, etc., are charged with the same amount of carbonate buffer or PBS. The plate is incubated for 40 minutes at 37°C (or overnight at 4°C) in a humid chamber, ~~washed in tap water,~~ and 300 ~~50~~ µl of blocking buffer is added to each well. The plate is incubated for 1 hour at ambient temperature, ~~20 minutes,~~ washed ~~in tap water, followed by~~ three times ~~wash cycles~~ with PBST, with soaking times of 3 minutes/cycle.

ii) 150 ~~50~~ µl of test samples and equine control sera prediluted 1/100 in sample/conjugate buffer is added in parallel to wells with and without antigen. The plate is incubated for 1 hour ~~30 minutes~~, washed ~~in tap water followed by~~ three times ~~wash cycles~~ with PBST.

iii) Properly diluted conjugate in sample/conjugate buffer is added in volumes of 150 ~~50~~ µl to all wells. The plate is incubated for 1 hour ~~30 minutes~~ with subsequent washing as above.

iv) 150 ~~100~~ µl of substrate indicator system is added to all wells and incubated for 1 hour.

v) The plate ~~reaction~~ is ~~stopped after 15 minutes at room temperature by the addition of 25 µl of 37 mM NaCN. Alternatively, commercially available detergents can be used after~~ pretesting shaken for 10 seconds, and the results are read photometrically at a wavelength of 415 ~~405~~ nm.

vi) *Calculation of results:* absorbance (with antigen) minus absorbance (without antigen) = net extinction. A reaction exceeding a net extinction of 0.3 is regarded as a positive result.

Standard positive and negative control sera should be included in each batch of tests.

A competitive ELISA has also been described for detecting antibody against *T. equiperdum* (Katz *et al.*, 2000).

d) Other serological tests

Other serological tests have been used, including radioimmunoassay, counter immunoelectrophoresis and agar gel immunodiffusion (AGID) tests (Caporale *et al.*, 1981; Hagebock *et al.*, 1993). The AGID has been used to confirm positive tests and to test anticomplementary sera. A seven-well pattern in 0.8% agarose in Tris buffer is used, with the CF test antigen in the centre well and positive control sera and unknown sera in alternate peripheral wells. A method has been published for diagnosing equine piroplasmosis, glanders and dourine at the same time, using immunoblotting (Katz *et al.*, 1999). A card agglutination test has been developed that compares favourably with the CF test (Claes *et al.*, 2005).

3. Confirmed case of dourine

In case of a serologically positive result and after clinical examination: repeat serological tests two times at 15 to 20 days of interval and perform an accurate epidemiological investigation.

A confirmed case of dourine is defined as an animal having a positive result with CFT or IFA or PCR and (i) showing clinical signs compatible with dourine or (ii) showing an increase in serological CFT titre in two consecutive tests or (iii) epidemiologically linked with a confirmed case of dourine (Calistri *et al.*, 2013).

C. REQUIREMENTS FOR VACCINES

~~There are~~ No vaccines ~~biological products~~ are available for this ~~Control of the~~ disease ~~depends on compulsory notification and slaughter of infected animals. Good hygiene at assisted matings is also essential~~.

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NB: There is an OIE Reference Laboratory for Dourine
(see Table in Part 4 of this *Terrestrial Manual* or consult the OIE web site for the most up-to-date list:
http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/ http://www.oie.int/).
Please contact the OIE Reference Laboratories for any further information on
diagnostic tests and reagents for dourine

CHAPTER 2.5.5.

EQUINE ENCEPHALOMYELITIS
(Eastern and Western)

SUMMARY

Eastern and Western equine encephalomyelitis viruses belong to the genus Alphavirus *of the family* Togaviridae*. Alternate infection of birds and mosquitoes maintain these viruses in nature. The disease occurs sporadically in horses and humans from mid-summer to late autumn. Horses and humans are tangential dead-end hosts. The disease in horses is characterised by fever, anorexia, and severe depression. In severe cases, the disease in horses progresses to hyperexcitability, blindness, ataxia, severe mental depression, recumbency, convulsions, and death. Eastern equine encephalomyelitis* (*EEE*) *virus infection in horses is often fatal, while Western equine encephalomyelitis* (*WEE*) *virus can cause a subclinical or mild disease with less than 30% mortality. EEE and WEE have been reported to cause disease in poultry, game birds and ratites. Sporadic cases of EEE have been reported in cows, sheep, pigs, deer, and dogs.*

Identification of the agent: *A presumptive diagnosis of EEE or WEE can be made when susceptible horses display the characteristic somnolence and other signs of neurological disease in areas where haematophagous insects are active. There are no characteristic gross lesions. Histopathological lesions can provide a presumptive diagnosis. EEE virus can usually be isolated from the brain and sometimes other tissues of dead horses, however WEE virus is rarely isolated. EEE and WEE viruses can be isolated from field specimens by inoculating newborn mice, embryonating chicken eggs, cell cultures, or newly hatched chickens. The virus is identified by complement fixation* (*CF*)*, immunofluorescence, or plaque reduction neutralisation* (*PRN*) *tests. EEE and WEE viral RNA may also be detected by reverse-transcription polymerase chain reaction methods.*

Serological tests: *Antibody can be identified by PRN, haemagglutination inhibition* (*HI*)*, CF tests, or IgM capture enzyme-linked immunosorbent assay.*

Requirements for vaccines ~~and diagnostic biologicals~~: *EEE and WEE vaccines are safe and immunogenic. They are produced in cell culture and inactivated with formalin.*

A. INTRODUCTION

Eastern equine encephalomyelitis (EEE) and Western equine encephalomyelitis (WEE) viruses are members of the genus *Alphavirus* of the family *Togaviridae*. The natural ecology for virus maintenance occurs via alternate infection of birds and ornithophilic mosquitoes. EEE virus has also been isolated from snakes, and these may have a role as reservoir hosts (Bingham *et al.*, 2012). Clinical disease may be observed in humans and horses, both of which are dead-end hosts for these agents. EEE has been diagnosed in Quebec and Ontario in Canada, central and eastern regions of the United States of America (USA), the Caribbean Islands, Mexico, and Central and South America. Disease caused by the WEE virus has been reported in the western USA and Canada, Mexico, and Central and South America (Morris, 1989; Reisen & Monath, 1989; Walton *et al*., 1981). Highlands J virus, antigenically related to WEE virus, has been isolated in eastern USA. Although Highlands J virus is generally believed not to cause disease in mammals, it has been isolated from the brain of a horse dying of encephalitis in Florida (Karabatsos *et al*., 1988).

Even though the mortality is lower for WEE, the clinical signs of EEE and WEE can be identical. The disease caused by either virus is also known as sleeping sickness. Following an incubation period of 5–14 days, clinical signs include fever, anorexia, and depression. A presumptive diagnosis of EEE or WEE virus infection in unvaccinated horses can be made if the characteristic somnolence is observed during the summer in temperate climates or the wet season in tropical and subtropical climates, when the mosquito vector is plentiful. However, a number of other diseases, such as West Nile virus and Venezuelan equine encephalomyelitis (chapters 2.1.20 and 2.5.13, respectively), produce similar clinical signs and the diagnosis must be confirmed by the described

diagnostic test methods. WEE virus infection in horses is often observed over a wide geographical area, e.g. sporadic cases over 1000 square miles. EEE virus infections are usually observed in limited geographical areas. Isolated events of high mortality in captive-raised game birds, primarily pheasants, chukars, aquarium penguins, and quail have been traced to WEE, EEE, or Highlands J virus infection (Morris, 1989; Reisen & Monath, 1989; Tuttle *et al*., 2005). Most encephalomyelitis infections in domestic fowl are caused by EEE virus and occur on the east coast states of the USA. The virus is introduced by mosquitoes, but transmission within the flocks is primarily by feather picking and cannibalism. Both EEE and WEE viruses have caused a fatal disease in ratites. Haemorrhagic enteritis has been observed in emus infected with EEE and WEE viruses, and morbidity and mortality rates may be greater than 85%. Highlands J and EEE viruses have been found to produce depression, somnolence, decreased egg production, and increased mortality in turkeys (Guy, 1997). EEE virus has been reported to cause disease in cows (McGee *et al*., 1992; Pursell *et al*., 1976), sheep (Bauer *et al*., 2005), pigs (Elvinger *et al*., 1996), white-tailed deer (Tate *et al*., 2005), and dogs (Farrar *et al*., 2005).

EEE virus causes severe disease in humans with a mortality rate of 30–70% and a high frequency of permanent sequelae in patients who survive. WEE is usually mild in adult humans, but can be a severe disease in children. The fatality rate is between 3 and 14%. Severe infection and death caused by EEE and WEE viruses have been reported in laboratory workers. ~~therefore, any work with these viruses must be performed at containment level 3 (see Chapter 1.1.3 *Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities*).~~ Laboratory manipulations should be carried out at an appropriate biosafety and containment level determined by biorisk analysis (see Chapter 1.1.3 *Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities*). It is recommended that personnel be immunised against EEE and WEE viruses (United States Department of Health and Human Services, 1999). Precautions should also be taken to prevent human infection when performing post-mortem examinations on horses suspected of being infected with the equine encephalomyelitis viruses.

B. DIAGNOSTIC TECHNIQUES

Table 1. *Test methods available for the diagnosis of eastern equine encephalomyelitis and their purpose**

Method	Purpose				
	Population freedom from infection	Individual animal freedom from infection	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
RT-PCR	–	++	+++	–	–
Isolation in tissue culture	–	++	+++	–	–
Immunohisto-chemistry	–	++	+++	–	–
IgM Capture ELISA	–	+	++	–	–
Plaque reduction neutralisation	+++	+	++	+++	+++
Hemagglutination inhibition (paired samples)	+	++	++	++	++
Complement fixation (paired samples)	–	+	++	–	–

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose, although not all of the tests listed as category +++ or ++ have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.
RT-PCR = reverse-transcription polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay.

*Although diagnostic methods for western equine encephalomyelitis are similar, not all test modalities have been thoroughly evaluated in horses naturally infected with WEE.

1. Identification of the agent

The definitive method for diagnosis of EEE or WEE is the isolation of the viruses. EEE virus can usually be isolated from the brains of horses, unless more than 5 days have elapsed between the appearance of clinical signs and the death of the horse. EEE virus can frequently be isolated from brain tissue even in the presence of a high serum antibody titre. WEE virus is rarely isolated from tissues of infected horses. Brain is the tissue of choice for virus isolation, but the virus has been isolated from other tissues, such as the liver and spleen. It is recommended that a complete set of these tissues be collected in duplicate, one set for virus isolation and the other set in formalin for histopathological examination. Specimens for virus isolation should be sent refrigerated if they can be received in the laboratory within 48 hours of collection; otherwise, they should be frozen and sent with dry ice. A complete set of tissues will allow the performance of diagnostic techniques for other diseases. For isolation, a 10% suspension of tissue is prepared in phosphate buffered saline (PBS), pH 7.8, containing bovine serum albumin (BSA) (fraction V; 0.75%), penicillin (100 units/ml), and streptomycin (100 µg/ml). The suspension is clarified by centrifugation at 1500 ***g*** for 30 minutes.

EEE and WEE viruses can be isolated in a number of cell culture systems. The most commonly used cell cultures are primary chicken or duck embryo fibroblasts, continuous cell lines of African green monkey kidney (Vero), rabbit kidney (RK-13), or baby hamster kidney (BHK-21). Isolation is usually attempted in 25 cm^2 cell culture flasks. Confluent cells are inoculated with 1.0 ml of tissue suspension. Following a 1–2-hour absorption period, maintenance medium is added. Cultures are incubated for 6–8 days, and one blind passage is made. EEE and WEE viruses will produce a cytopathic change in cell culture. Cultures that appear to be infected are frozen. The fluid from the thawed cultures is used for virus identification.

The newborn mouse is also considered to be a sensitive host system. Inoculate intracranially one or two litters of 1–4-day-old mice with 0.02 ml of inoculum using a 26-gauge 3/8 inch (9.3 mm) needle attached to a 1 ml tuberculin syringe. The inoculation site is just lateral to the midline into the midportion of one lateral hemisphere. Mice are observed for 10 days. Mice that die within 24 hours of inoculation are discarded. From 2 to 10 days postinoculation, dead mice are collected daily and frozen at –70°C. Mouse brains are harvested for virus identification by aspiration using a 20-gauge 1 inch (2.5 cm) needle attached to a 1 ml tuberculin syringe. A second passage is made only if virus cannot be identified from mice that die following inoculation.

The chicken embryo is considered to be less sensitive than newborn mice when used for primary isolation of EEE and WEE viruses. Tissue suspensions can be inoculated by the yolk-sac route into 6–8-day-old embryonating chicken eggs. There are no diagnostic signs or lesions in the embryos infected with these viruses. Inoculated embryos should be incubated for 7 days, but deaths usually occur between 2 and 4 days post-inoculation. Usually only one passage is made unless there are dead embryos from which virus cannot be isolated. Newly hatched chickens are susceptible and have been used for virus isolation. If this method is used, precautions must be taken to prevent aerosol exposure of laboratory personnel, as infected birds can shed highly infectious virus.

EEE or WEE viruses can be identified in infected mouse or chicken brains, cell culture fluid, or amnionic-allantoic fluid by complement fixation. A 10% brain suspension is prepared in veronal (barbitone) buffer; egg and cell culture fluids are used undiluted or diluted 1/10 in veronal buffer. The fluid or suspension is centrifuged at 9000 ***g*** for 30 minutes, and the supernatant fluid is tested against hyperimmune serum or mouse ascitic fluid prepared against EEE and WEE viruses using a standard CF procedure (United States Department Of Health, Education and Welfare, 1974). The CF test requires the overnight incubation at 4°C of serum-antigen with 7 units of complement. Virus can be identified in cell culture by direct immunofluorescent staining. The less commonly used method of virus identification is the neutralisation test, as outlined below.

Reverse-transcription polymerase chain reaction (RT-PCR) methods to detect EEE, WEE and VEE viral nucleic acid in mosquitoes and vertebrate tissues have been described, although few have been extensively validated for mammalian samples (Lambert *et al*., 2003; Linssen *et al*., 2000; Monroy *et al*., 1996; Vodkin *et al*., 1993). A multiplex RT-PCR method was developed to expedite differential diagnosis in cases of suspected EEE or West Nile arboviral encephalomyelitis in horses (Johnson *et al*., 2003). The assay has enhanced speed and sensitivity compared with cell culture virus isolation and has been used effectively in the USA during several recent arbovirus seasons. Recently, a combination of an RT-PCR with an enzyme-linked immunosorbent assay (ELISA: RT-PCR-ELISA) was reported as a method to identify alpha-viruses that are pathogenic to humans (Wang *et al*., 2006).

Antigen-capture ELISA has been developed for EEE surveillance in mosquitoes. This can be used in countries that do not have facilities for virus isolation or RT-PCR (Brown *et al*., 2001). Immunohistochemical (IHC) procedures are very useful for diagnosis of EEE as they are carried out on fixed tissues ~~have also been described (Patterson~~ Pennick *et al*., 2012~~ 1996~~). The envelope protein of EEEV is targeted in IHC. Necrotic and inflamed areas of the brain are examined. In EEEV infected horses, positive staining is observed most notably in neurons and associated dendritic processes.

2. Serological tests

Serological confirmation of EEE or WEE virus infection requires a four-fold or greater increase or decrease in antibody titre in paired serum samples collected 10–14 days apart. Most horses infected with EEE or WEE virus have a high antibody titre when clinical disease is observed. Consequently, a presumptive diagnosis can be made if an unvaccinated horse with appropriate clinical signs has antibody against only EEE or WEE virus. The detection of IgM antibody by the ELISA can also provide a presumptive diagnosis of acute infection (Sahu *et al*., 1994). The plaque reduction neutralisation (PRN) test or, preferably, a combination of PRN and haemagglutination inhibition (HI) tests is the procedure most commonly used for the detection of antibody against EEE and WEE viruses. There may be cross-reactions between antibody against EEE and WEE virus in the CF and HI tests. CF antibody against both EEE and WEE viruses appears later and does not persist; consequently, it is less useful for the serological diagnosis of disease.

a) Complement fixation

The CF test is frequently used for the demonstration of antibodies, although the antibodies detected by the CF test may not persist for as long as those detected by the HI or PRN tests. A sucrose/acetone mouse brain extract is commonly used as antigen. The positive antigen is inactivated by treatment with 0.1% beta-propiolactone.

In the absence of an international standard serum, the antigen should be titrated against a locally prepared positive control serum. The normal antigen, or control antigen, is mouse brain from uninoculated mice similarly extracted and diluted.

Sera are diluted 1/4 in veronal buffered saline containing 1% gelatin (VBSG), and inactivated at 56°C for 30 minutes. Titrations of positive sera may be performed using additional twofold dilutions. The CF antigens and control antigen (normal mouse brain) are diluted in VBSG to their optimal amount of fixation as determined by titration against the positive sera; guinea-pig complement is diluted in VBSG to contain 5 complement haemolytic units-50% (CH_{50}). Sera, antigen, and complement are reacted in 96-well round-bottom microtitre plates at 4°C for 18 hours. The sheep red blood cells (SRBCs) are standardised to 2.8% concentration. Haemolysin is titrated to determine the optimal dilution for the lot of complement used. Haemolysin is used to sensitise 2.8% SRBCs and the sensitised cells are added to all wells on the microtitre plate. The test is incubated for 30 minutes at 37°C. The plates are then centrifuged (200 ***g***), and the wells are scored for the presence of haemolysis. The following controls are used: (a) serum and control serum each with 5 CH_{50} and 2.5 CH_{50} of complement; (b) CF antigen and control antigen each with 5 CH_{50}, and 2.5 CH_{50} of complement; (c) complement dilutions of 5 CH_{50}, 2.5 CH_{50}, and 1.25 CH_{50}; and (d) cell control wells with only SRBCs and VBSG diluent. These controls test for anticomplementary serum and anticomplementary antigen, activity of complement used in the test, and integrity of the SRBC indicator system in the absence of complement, respectively.

To avoid anticomplementary effects, sera should be separated from the blood as soon as possible. Positive and negative control sera should be used in the test.

b) Haemagglutination inhibition

The antigen for the HI test is the same as described above for the CF test. The antigen is diluted so that the amount used in each haemagglutinating unit (HAU) is from four to eight times that which agglutinates 50% of the RBCs in the test system. The haemagglutination titre and optimum pH for each antigen are determined with goose RBCs diluted in pH solutions ranging from pH 5.8 to pH 6.6, at 0.2 intervals.

Sera are diluted 1/10 in borate saline, pH 9.0, and then inactivated at 56°C for 30 minutes. Kaolin treatment is used to remove nonspecific serum inhibitors. Alternatively, nonspecific inhibitors may be removed by acetone treatment of serum diluted 1/10 in PBS followed by reconstitution in borate saline. Sera should be absorbed before use by incubation with a 0.05 ml volume of washed packed goose RBCs for 20 minutes at 4°C.

Following heat inactivation, kaolin treatment and absorption, twofold dilutions of the treated serum are prepared in borate saline, pH 9.0 with 0.4% bovalbumin. Serum dilutions (0.025 ml/well) are prepared in a 96-well round-bottom microtitre plate in twofold dilutions in borate saline, pH 9.0, with 0.4% bovalbumin. Antigen (0.025 ml/well) is added to the serum. Plates are incubated at 4°C overnight. RBCs are derived from normal white male geese [1] and washed three times in dextrose/gelatin/veronal (DGV), and a 7.0% suspension is prepared in DGV. The 7.0% suspension is then diluted 1/24 in the appropriate pH solution, and 0.05 ml per well is added immediately to the plates. Plates are incubated for 30 minutes at 37°C. Positive and negative control sera are incorporated into each test. A test is considered to be valid only if the control sera give the expected results. Titres of 1/10 and 1/20 are suspect, and titres of 1/40 and above are positive.

1 RBCs from adult domestic white male geese are preferred, but RBCs from other male geese can be used. If cells from female geese are used, there may be more test variability. It has been reported that rooster RBCs cause a decrease in the sensitivity of the test.

c) Enzyme-linked immunosorbent assay

The ELISA is performed by coating flat-bottomed plates with anti-equine IgM capture antibody (Sahu *et al*., 1994). The antibody is diluted according to the manufacture's recommendations in 0.5 M carbonate buffer, pH 9.6, and 50 µl is added to each well. The plates are incubated at 37°C for 1 hour, and then at 4°C overnight. Prior to use, the coated plates are washed three times with 200–300 µl/well of 0.01 M PBS containing 0.05% Tween 20. After the second wash, 200 µl/well of PBS/Tween/5% nonfat dried milk is added and the plates are incubated at room temperature for 1 hour. Following incubation, the plates are washed again three times with PBS/Tween. Test and control sera are diluted 1/400 in 0.01 M PBS, pH 7.2, containing 0.05% Tween 20, and 50 µl is added to each well. The plates are incubated at 37°C for 90 minutes and then washed three times. Next, 50 µl of viral antigen is added to all wells. (The dilution of the antigen will depend on the source and should be empirically determined.) The plates are incubated overnight at 4°C, and washed three times. Then, 50 µl of horseradish-peroxidase-conjugated monoclonal antibody (MAb) to encephalitis virus[2] is added. The plates are incubated for 90 minutes at 37°C and then washed six times. Finally, 50 µl of freshly prepared ABTS (2,2'-Azino-bis-[3-ethylbenzo-thiazoline-6-sulphonic acid]) substrate + hydrogen peroxidase is added, and the plates are incubated at room temperature for 15–40 minutes The absorbance of the test serum is measured at 405 nm. A test sample is considered to be positive if the absorbance of the test sample in wells containing virus antigen is at least twice the absorbance of negative control serum in wells containing virus antigen and at least twice the absorbance of the sample tested in parallel in wells containing normal antigen.

d) Plaque reduction neutralisation

The PRN test is very specific and can be used to differentiate between EEE and WEE virus infections. The PRN test is performed in duck embryo fibroblast, Vero, or BHK-21 cell cultures in 25 cm^2 flasks or six-well plates. Volumes listed are for flasks; the volume should be halved for wells in six-well plates. The sera can be screened at a 1/10 and 1/100 final dilution. Endpoints can be established using the PRN or HI test. Serum used in the PRN assay is tested against 100 plaque-forming units (PFU) of virus (50 PFU for six-well plates). The virus/serum mixture is incubated at 37°C for 75 minutes before inoculation on to confluent cell culture monolayers in 25 cm^2 flasks. The inoculum is adsorbed for 1 hour, followed by the addition of 6 ml of overlay medium. The overlay medium consists of two solutions that are prepared separately. Solution I contains 2 × Earle's Basic Salts Solution without phenol red, 4% fetal bovine serum, 100 µg/ml gentamicin, 200 µg/ml nystatin, 0.45% solution of sodium bicarbonate, and 0.002% neutral red. When duck embryo fibroblasts are used, Solution 1 also contains 6.6% yeast extract lactalbumin hydrolysate. Solution II consists of 2% Noble agar that is sterilised and maintained at 47°C. Equal volumes of solutions I and II are adjusted to 47°C and mixed together just before use. The test is incubated for 48–72 hours, and endpoints are based on a 90% reduction in the number of plaques compared with the virus control flasks, which should have about 100 plaques.

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

1. Background

Inactivated vaccines against EEE and WEE viruses are available commercially. Attenuated EEE and WEE virus vaccines have not proven satisfactory. The vaccines licensed for use in the USA are prepared using the following combinations: EEE and WEE; EEE, WEE, and Venezuelan equine encephalomyelitis (VEE); and EEE and VEE. In addition, tetanus toxoid, inactivated influenza virus, and inactivated West Nile virus have been combined with EEE and WEE or EEE, WEE, and VEE. ~~Early vaccines were produced from virus propagated in embryonating chicken eggs and inactivated with formalin.~~ Current vaccines are prepared from virus propagated in cell culture, and inactivated with formalin (Maire *et al*., 1970) ~~or monoethylamine~~.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 *Principles of veterinary vaccine production*. The guidelines given below and in chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements

~~1. Seed management~~

2 Available from: Centers for Disease Control and Prevention, Biological Reference Reagents, 1600 Clifton Road NE, Mail Stop C21, Atlanta, Georgia 30333, United States of America.

2. Outline of production and minimum requirements for vaccines ~~Seed management~~[3]

a) Characteristics of the seed

See chapter 1.1.6 *Principles of veterinary vaccine production*, for general requirements for Master Seeds and allowable passages for vaccine production. Suitable seed lots should be maintained at –70°C in a lyophilised state.

i) *Biological characteristics of the master seed*

Standard strains of EEE and WEE viruses were isolated over 20 years ago, have been used for vaccine production and have been proven to produce a protective immunity. Strains of EEE virus that differ antigenically and in molecular structure have been identified from different geographical regions. However, the North American and Caribbean isolates appear to be similar (Weaver *et al*., 1994). Strains of WEE virus isolated from different countries have been found to be similar both by MAb testing and RNA oligonucleotide fingerprinting analysis (Reisen & Monath, 1989). A recent well-characterised isolate from the country where the vaccine is to be used would be advantageous. Selected viruses must be immunogenic and replicate to high titres in cell culture.

ii) *Quality criteria* (*sterility, purity, freedom from extraneous agents*)

The MSV must be tested for purity, identity, and freedom from extraneous agents at the time before it is used in the manufacture of vaccine. The MSV must be free from bacteria, fungi and mycoplasma. The MSV is cultured on a Vero cell line and an embryonic equine cell type with confirmation by the fluorescent antibody technique to demonstrate freedom from equine herpesvirus, equine adenovirus, equine arteritis virus, bovine viral diarrhoea virus, reovirus, and rabies virus extraneous agents The MSV must also be free from extraneous virus by cytopathic effect (CPE) and haemadsorption on cell culture on the Vero cell line and an embryonic equine cell type.

iii) *Validation as a vaccine strain*

In an immunogenicity trial, the MSV at the highest passage level intended for production must prove its efficacy (protection) in the guinea-pig vaccination/serology potency test.

iv) *Emergency procedure for provisional acceptance of new master seed virus* (*MSV*) *in the case of an epizootic* (*with pathogens with many serotypes, e.g. bluetongue virus, highly pathogenic avian influenza, FMD, etc*.)

In an emergency epizootic situations, there may be not enough time to fully test a new MSV for all extraneous agents; in such a situation, provisional acceptance of the new strain could be based on a risk analysis of the possibility of contamination of the antigen produced from the new MSV with extraneous agents. This risk assessment should take into account the characteristics of the process, including the nature and concentration of the inactivant for inactivated vaccines, before allowing or not the early release of the new product

~~b) Method of culture~~

~~Primary chicken embryo fibroblasts and Vero cells have been used for propagation of viruses used for vaccine production. The fibroblasts should be prepared from specific pathogen free embryos. Other susceptible cell lines could also be used.~~

~~c) Validation as a vaccine~~

~~If a cell line is used, the master cell stock is tested to confirm the identity of the cell line, species of origin, and freedom from extraneous agents. If primary cell cultures are used, a monolayer from each batch of each subculture should be tested for extraneous agents including bacteria, fungi, mycoplasma, and viruses. The master seed virus should also be tested to ensure freedom from bacteria, fungi, mycoplasma, and extraneous viruses (see Chapter 1.1.7 *Tests for sterility and freedom from contamination of biological materials for a description of the method*).~~

~~The vaccines are administered by the intramuscular (in most cases) or intradermal route in the cervical region in two doses given 2–4 weeks apart. Annual revaccination is recommended. All foals vaccinated before 1 year of age should be revaccinated before the next vector season.~~

3 Sections on Seed management, Manufacture, In-process control, and Batch control are taken from the Biotechnology, Biologics, and Environmental Protection Division of the United States Department of Agriculture's Animal and Plant Health Inspection Service (APHIS).

b) Method of manufacture

i) *Procedure*

The MSV should be propagated in cell lines known to support the growth of EEE and WEE. See chapter 1.1.6 for additional guidance on the preparation and testing of master cell stocks. Cell lines should be free from extraneous viruses, bacteria, fungi, and mycoplasma. Viral propagation should not exceed five passages from the MSV, unless further passages prove to provide sufficient serological titres in guinea-pigs.

The susceptible cell line is seeded into suitable vessels. Minimal essential medium, supplemented with fetal bovine serum (FBS), may be used as the medium for production. Incubation is at 37°C.

Cell cultures are inoculated directly with EEE and WEE working virus stock, which is generally 1 to 4 passages from the MSV. Inoculated cultures are incubated for 1–3 days before harvesting the culture medium. During incubation, the cultures are observed daily for CPE and bacterial contamination.

Inactivated vaccines may be chemically inactivated with formalin and mixed with a suitable adjuvant. The duration of the inactivation period is based on demonstrated inactivation kinetics.

The preservatives used are thimerosal at a 1/1000 dilution and antibiotics (neomycin, polymyxin, amphotercin B, and gentamicin).

ii) *Requirements for ingredients*

All ingredients used in the manufacture of EEE and WEE vaccine should be defined in approved manufacturing protocols and consistent from batch to batch. See chapter 1.1.6 for general guidance on ingredients of animal origin. Ingredients of animal origin should be sourced from a country with negligible risk for transmissible spongiform encephalopathies (TSEs).

iii) *In-process controls*

Production lots should be examined daily for cytopathic changes. After harvesting, the virus suspension should be tested for the presence of microbial contaminants. Production lots must be titrated in tissue culture before inactivation to standardise the product. Low-titred lots may be concentrated or blended with higher-titred lots to achieve the correct titre.

Inactivated lots must be tested for completeness of inactivation in 6- to 12-hour old chicks.

iv) *Final product batch tests*

Sterility

Inactivated and live vaccine samples are examined for bacterial and fungal contamination. The volume of medium used in these tests should be enough to nullify any bacteriostatic or fungistatic effects of the preservatives in the product. To test for bacteria, ten vessels, each containing a minimum of 120 ml of soybean casein digest medium, are inoculated with 0.2 ml from ten final-container samples. The ten vessels are incubated at 30–35°C for 14 days and observed for bacterial growth. To test for fungi, ten vessels, each containing a minimum of 40 ml soybean casein digest medium, are inoculated with 0.2 ml from ten final-container samples. The vessels are incubated at 20–25°C for 14 days and observed for fungal growth. Individual countries may have other requirements.

Identity

Separate batch tests for identity should be conducted if the batch potency test, such as tissue-culture titrations of live virus vaccines, does not sufficiently verify the identity of the agent in the vaccine. Identity tests may include fluorescent antibody or serum neutralisation assays.

Safety

Batch safety testing for virus inactivation is conducted in 6- to 12-hour old chicks. Ten chicks are inoculated subcutaneously with 0.5 ml of product and observed daily for 10 days. If unfavourable reactions occur, the batch is unacceptable.

Batch potency

Potency testing is performed by inoculating each of ten guinea-pigs with either EEE or WEE virus vaccine stock, using one-half the horse dose on two occasions, 14–21 days apart, by the route recommended for the horse. Serum samples from each vaccinate and each control are tested 14–21 days after the second dose using the PRN test. The EEE titres should be ≥1/40, and the WEE titres should be ≥1/40 (US Code of Federal Regulations), using Vero cells. If duck embryo fibroblasts are used in the PRN test, the titres will be lower. An alternative potency test is to use intracerebral challenge, 14–21 days after the second vaccination. Each guinea pig is inoculated with 0.1 ml of virus

containing 100 LD_{50} (50% lethal dose). Simultaneous titration is carried out. In order for the vaccine to be approved, 80% of the guinea pigs must survive both viruses.

c) Requirements for authorisation/registration/licensing

i) *Manufacturing process*

For registration of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Section C.2.a and b) should be submitted to the authorities. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

In-process controls are part of the manufacturing process.

ii) *Safety requirements*

The final inactivated vaccine formulation should be tested in a limited number of target animals prior to a larger-scale field study. The final vaccine formulation should not cause adverse reactions.

Field safety studies should be conducted before any vaccine receives final approval. Generally, two serials should be used, in three different geographical locations under typical animal husbandry conditions, and in a minimum of 600 animals. The vaccine should be administered according to label recommendations (including booster doses) and should contain the maximum permissible amount of viral antigen. (If no maximum antigen content is specified, serials should be of anticipated typical post-marketing potency.) About one-third of the animals should be at the minimum age recommended for vaccination.

- *Precautions* (*hazards*)

Vaccine should be identified as harmless or pathogenic for vaccinators. Manufacturers should provide adequate warnings that medical advice should be sought in the case of self-injection (including for adjuvants, oil-emulsion vaccine, preservatives, etc.) with warnings included on the product label/leaflet so that the vaccinator is aware of any danger.

iii) *Efficacy requirements*

To register a commercial vaccine, a batch or batches produced according to the standard method and containing the minimum amount of antigen or potency value shall prove its efficacy (protection) in the guinea-pig vaccination/serology potency test; each future commercial batch shall be tested before release to ensure it has the same potency value demonstrated by the batch(es) used for the efficacy test(s).

iv) *Vaccines permitting a DIVA strategy* (*detection of infection in vaccinated animals*)

Vaccinated horses may develop a serological titre which may interfere with the ability to export the horse.

~~Details of the manufacture of vaccines currently on the market are not available. Consequently, the information provided here is intended only as background reference material on the vaccines and not as a method of manufacture. The virus and cell culture system should be selected so that a high virus titre, $\geq 10^6$ $TCID_{50}$ (50% tissue culture infective dose) per ml, is obtained in under 48 hours. Virus for vaccine production can be prepared from the supernatant fluid from infected cell cultures. The fluid is harvested when 70–100% of the monolayers have the characteristic cytopathic changes. The virus titre is determined by titration in cell culture or mice. The fluid is clarified by low speed centrifugation and filtered through gauze. The virus is inactivated by adding formalin to a final concentration of 1/2000 (0.05%) and holding at 37°C for 24 hours. Residual formaldehyde is neutralised by sodium bisulphite (Maire *et al.*, 1970). The residual free formalin content in the inactivated vaccine should not exceed 0.2% formaldehyde.~~

~~**3. In-process control**~~

~~Cultures should be examined daily for virus induced cytopathic effect. The harvested virus should be tested for microbial contamination. The efficacy of the inactivation process should be checked by testing for viable virus.~~

~~**4. Batch control**~~

~~**a) Sterility**~~

~~Tests for sterility and freedom from contamination of biological materials may be found in chapter 1.1.7.~~

~~**b) Safety**~~

~~The inactivated vaccine is safety tested by inoculating subcutaneously at least ten 6- to 12-hour-old chickens with 0.5 ml of the vaccine. The chickens are observed each day for 10 days for unfavourable reactions that are attributable to the vaccine (United States Code of Federal Regulations, 2000). Safety testing can also be carried out by inoculating intracerebrally at least eight 1–4 day old mice with 0.02 ml of the vaccine, and observing for 7 days. It is critical that safety tests be conducted on each lot of vaccine to insure that there is no residual virulent virus present.~~

~~c) Potency~~

~~Potency testing is performed by inoculating each of ten guinea pigs with either EEE or WEE virus, using one-half the horse dose on two occasions, 14–21 days apart, by the route recommended for the horse. Serum samples from each vaccinate and each control are tested 14–21 days after the second dose using the PRN test. The EEE titres should be ≥1/40, and the WEE titres should be ≥1/40 (US Code of Federal Regulations), using Vero cells. If duck embryo fibroblasts are used in the PRN test, the titres will be lower. An alternative potency test is to use intracerebral challenge, 14–21 days after the second vaccination. Each guinea-pig is inoculated with 0.1 ml of virus containing 100 LD_{50} (50% lethal dose). Simultaneous titration is carried out. In order for the vaccine to be approved, 80% of the guinea pigs must survive both viruses.~~

~~d) Duration of immunity~~

v) *Duration of immunity*

Comprehensive studies on duration of immunity are not available. An annual revaccination is recommended for the inactivated vaccine. Foals ~~that are~~ vaccinated under 1 year of age should be revaccinated before the next vector season.

~~e) Stability~~

vi) *Stability*

The inactivated ~~lyophilised~~ vaccine is stable and immunogenic for 2 ~~3~~ years if kept refrigerated at 2–7°C. After 2 ~~3~~ years, vaccine should be discarded. ~~The vaccines should be used immediately after reconstitution.~~

~~f) Preservatives~~

~~The preservatives used are thimerosal at a 1/10,000 dilution and antibiotics (neomycin, polymyxin amphotercin B and gentamicin).~~

~~g) Precautions (hazards)~~

~~Severe infection and death caused by EEE and WEE viruses have been reported in laboratory workers; therefore, any work with these viruses must be carried out at least in a containment level 3 laboratory (see chapter 1.1.3) using biological safety cabinets, and it is recommended that personnel be immunised against EEE and WEE viruses (United States Department of Health and Human Services, 1999).~~

~~Pregnant mares and foals under 2 weeks old should not be vaccinated.~~

~~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 is an OIE Reference Laboratory for Equine encephalomyelitis (Eastern and Western):
(see Table in Part 4 of this *Terrestrial Manual* or consult the OIE web site for the most up-to-date list:
http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/ http://www.oie.int/).
Please contact the OIE Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for equine encephalomyelitis (Eastern and Western)

CHAPTER 2.5.6.

EQUINE INFECTIOUS ANAEMIA

SUMMARY

Equine infectious anaemia (*EIA*) *is a persistent viral infection of equids. The causative agent, EIA virus* (*EIAV*) *is a lentivirus in the family* Retroviridae*, subfamily* Orthoretrovirinae*. Other members of the genus* Lentivirus *include: bovine immunodeficiency virus; caprine arthritis encephalitis virus; feline immunodeficiency virus; human immunodeficiency virus 1; human immunodeficiency virus 2; and maedi/visna virus. EIA can be diagnosed on the basis of clinical signs, pathological lesions, serology and molecular methods. Infected horses remain viraemic carriers for life and, with very rare exceptions, yield a positive serological test result. Antibody response usually persists and antibody-positive animals, older than 6–8 months, are identified as virus carriers* (*below 6–8 months of age, serological reactions can be due to maternal antibodies; status can be confirmed by molecular techniques*)*. Infected equids are potential virus reservoirs. Biting flies are mechanical vectors for the virus in nature.*

Identification of the agent: *Virus from a horse can be isolated by inoculating suspect blood into a susceptible horse or on to leukocyte cultures prepared from susceptible horses. Recognition of infection in horses that have been inoculated experimentally may be made on the basis of clinical signs, haematological changes and a positive antibody response determined by an immunodiffusion test or enzyme-linked immunosorbent assay* (*ELISA*) *or by molecular techniques. Successful virus isolation in horse leukocyte cultures is confirmed by the detection of specific EIA antigen, by immunofluorescence assay, polymerase chain reaction, reverse-transcriptase assay, or by the inoculation of culture fluids into susceptible horses. Virus isolation is rarely attempted because of the time, difficulty and expense involved.*

Serological tests: *Agar gel immunodiffusion* (*AGID*) *tests and ELISAs are simple, reliable tests for the demonstration of EIAV infection. When ELISAs are positive they should be confirmed using the AGID test. EIA antigens can be prepared from infected tissue cultures or by using recombinant DNA technology.*

Requirements for vaccines ~~and diagnostic biologicals~~: *An attenuated live vaccine, developed in the early 1970s, was extensively used in China* (*People's Rep. of*) *between 1975 and 1990. Since then, the strategy for EIA control has shifted from vaccination to quarantine to avoid the interference of vaccinal antibodies with diagnostic tests. There are no ~~biological products~~ vaccines currently available.*

A. INTRODUCTION

Equine infectious anaemia (EIA) occurs world-wide. The infection, formerly known as swamp fever, is limited to equids. The disease is characterised by recurrent febrile episodes, thrombocytopenia, anaemia, rapid loss of weight and oedema of the lower parts of the body. If death does not result from one of the acute clinical attacks, a chronic stage develops and the infection tends to become inapparent. The incubation period is normally 1–3 weeks, but may be as long as 3 months. In acute cases, lymph nodes, spleen and liver are hyperaemic and enlarged. Histologically these organs are infiltrated with nests of immature lymphocytes and plasma cells. Kupffer cells in the liver often contain haemosiderin or erythrocytes. The enlarged spleen may be felt on rectal examination. Differential diagnoses include equine viral arteritis (Chapter 2.5.10), *Anaplasma phagocytophilum*, and other causes of oedema, fever, anaemia, or thrombocytopaenia/ecchymoses.

EIA virus (EIAV) is in the genus *Lentivirus* in the family *Retroviridae*, subfamily *Orthoretrovirinae*. Other members of the genus include: bovine immunodeficiency virus; caprine arthritis encephalitis virus; feline immunodeficiency virus; human immunodeficiency virus 1; human immunodeficiency virus 2; and maedi/visna virus. Nucleic acid sequence comparisons have demonstrated a marked relatedness among these viruses.

Once a horse is infected with EIAV, its blood remains infectious for the remainder of its life. This means that the horse is a viraemic carrier and can potentially transmit the infection to other horses (Cheevers & McGuire, 1985). Transmission occurs by transfer of blood from an infected horse. In nature, spread of the virus is most likely via interrupted feeding of bloodsucking horseflies on a clinically ill horse and then on susceptible horses. Transmission can also occur by the iatrogenic transfer of blood through the use of contaminated needles. *In utero* infection of the fetus may occur (Kemen & Coggins, 1972). The virus titre is higher in horses with clinical signs and the risk of transmission is higher from these animals than the carrier animals with a lower virus titre.

EIAV is not considered a risk for human health. Laboratory manipulations should be carried out at an appropriate biosafety and containment level determined by biorisk analysis (see Chapter 1.1.3 *Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities*).

B. DIAGNOSTIC TECHNIQUES

Agar gel immunodiffusion (AGID) tests (Coggins *et al*., 1972) and enzyme-linked immunosorbent assays (ELISAs) (Suzuki *et al*., 1982) are accurate, reliable tests for the detection of EIA in horses, except for animals in the early stages of infection and foals of infected dams. In other rare circumstances, misleading results may occur when the level of virus circulating in the blood during an acute episode of the disease is sufficient to bind available antibody, and if initial antibody levels never rise high enough to be detectable (Toma, 1980). Although the ELISA will detect antibodies somewhat earlier and at lower concentrations than the AGID test, positive ELISAs are confirmed using the AGID test. This is because false-positive results have been noted with ELISAs. The AGID test also has the advantage of distinguishing between EIA and non-EIA antigen–antibody reactions by lines of identity.

Table 1. *Test methods available for the diagnosis of equine infectious anaemia and their purpose*

Method	**Purpose**			
	Population freedom from infection/ efficiency of eradication policies	Individual animal freedom from infection	Confirmation of clinical cases	Prevalence of infection – surveillance
Agar gel immunodiffusion	++	++	++	++
Enzyme-linked immunosorbent assay	++	++	+	+
Immunoblot	=	++	++	=
Polymerase chain reaction	=	+/–	+	=
Virus isolation/horse inoculation	=	=	+	=

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose.
Although not all of the tests listed as category +++ or ++ have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

~~The EIAV is a *Lentivirus* in the family *Retroviridae*, subfamily *Orthoretrovirinae*. Other members of the lentivirus genus include: bovine immunodeficiency virus; caprine arthritis encephalitis virus; feline immunodeficiency virus; human immunodeficiency virus 1; human immunodeficiency virus 2; and maedi/visna virus. Nucleic acid sequence comparisons have demonstrated a marked relatedness among these viruses.~~

1. Identification of the agent

a) Virus isolation and identification

Virus isolation is usually not necessary to make a diagnosis.

Isolation of the virus from suspect horses may be made by inoculating their blood on to leukocyte cultures prepared from horses free of infection. Virus production in cultures can be confirmed by detection of specific EIA antigen by ELISA (Shane *et al*., 1984), by immunofluorescence assay (Weiland *et al*., 1982), by molecular tests or by subinoculation into susceptible horses. Virus isolation is rarely attempted because of the difficulty of growing horse leukocyte cultures.

When the exact status of infection of a horse cannot be ascertained, the inoculation of a susceptible horse with suspect blood ~~should~~ may be employed. In this case a horse that has previously been tested for antibody and shown to be negative is given an immediate blood transfusion from the suspect horse, and its antibody status and clinical condition are monitored for at least 45 days. Usually, 1–25 ml of whole blood given intravenously is sufficient to demonstrate infection, but in rare cases it may be necessary to use a larger volume of blood (250 ml) or washed leukocytes from such a volume (Coggins & Kemen, 1976).

b) Polymerase chain reaction

A nested polymerase chain reaction (PCR) assay to detect EIA proviral DNA from the peripheral blood of horses has been described (Nagarajan & Simard, 2001). The nested PCR method is based on primer sequences from the *gag* region of the proviral genome. It has proven to be a sensitive technique to detect field strains of EAV in white blood cells of EIA infected horses; the lower limit of detection is typically around 10 genomic copies of the target DNA (Nagarajan & Simard, 2001; 2007). A real-time reverse-transcriptase PCR assay has also been described (Cook *et al*., 2002). To confirm the results of these very sensitive assays, it is recommended that duplicate samples of each diagnostic specimen be processed. Because of the risk of cross contamination, it is also important that proper procedures are followed (see Chapter 1.1.4 *Quality management in veterinary testing laboratories* and ~~Methods to insure the validity of PCR testing are discussed in detail in~~ Chapter 1.1.5 *Principles and methods of validation of diagnostic assays for infectious diseases*) ~~Validation and quality control of polymerase chain reaction methods used for the diagnosis of infectious diseases~~.

The following are some of the circumstances where the PCR assay maybe used for the detection of EIAV infection in horses:

- Conflicting results on serologic tests;
- Suspected infection but negative or questionable serologic results;
- Complementary test to serology for the confirmation of positive results;
- Confirmation of early infection, before serum antibodies to EIAV develop;
- Ensuring that horses that are to be used for antiserum or vaccine production or as blood donors are free of EIAV;
- Confirmation of the status of a foal from an infected mare.

2. Serological tests

Due to the persistence of EIAV in infected equids, detection of serum antibody to EIAV confirms the diagnosis of EIAV infection.

a) Agar gel immunodiffusion test (the prescribed test for international trade)

Precipitating antibody is rapidly produced as a result of EIA infection, and can be detected by the AGID test. Specific reactions are indicated by precipitin lines between the EIA antigen and the test serum and confirmed by their identity with the reaction between the antigen and the positive standard serum. ~~Horses in the first 2–3 weeks after infection will usually give negative serological reactions. In rare cases the post-infection time prior to the appearance of detectable antibody may extend up to 60 days.~~

Reagents for AGID are available commercially from several companies. Alternately, AGID antigen and reference serum may be prepared as described below.

o Preparation of antigen

Specific EIA antigen may be prepared from the spleen of acutely infected horses (Coggins *et al*., 1973), from infected equine tissue culture (Malmquist *et al*., 1973), from a persistently infected canine thymus cell line (Bouillant *et al*., 1986), or from proteins expressed in bacteria or baculovirus using the recombinant DNA technique (Archambault *et al*., 1989; Kong *et al*., 1997). Preparation from infected cultures or from recombinant DNA techniques gives a more uniform result than the use of spleen cells and allows for better standardisation of reagents.

To obtain a satisfactory antigen from spleen, a horse must be infected with a highly virulent strain of EIAV. The resulting incubation period should be 5–7 days, and the spleen should be collected 9 days after

inoculation, when the virus titre is at its peak and before any detectable amount of precipitating antibody is produced. Undiluted spleen pulp is used in the immunodiffusion test as antigen (Coggins *et al*., 1973). Extraction of antigen from the spleen with a saline solution and concentration with ammonium sulphate does not give as satisfactory an antigen as selection of a spleen with a very high titre of EIA antigen.

Alternatively, equine fetal kidney or dermal cells or canine thymus cells are infected with a strain of EIAV adapted to grow in tissue culture (American Type Culture Collection, or Chinese strain adapted to equine fetal dermal cells). Virus is collected from cultures by precipitation with 8% polyethylene glycol or by pelleting by ultracentrifugation. The diagnostic antigen, p26, is released from the virus by treatment with detergent or ether (Malmquist *et al*., 1973). EIAV core proteins, expressed in bacteria or baculovirus, are commercially available and find practical use as high quality antigens for serological diagnosis.

The p26 is an internal structural protein of the virus that is coded for by the *gag* gene. The p26 is more antigenically stable among EIAV strains than the virion glycoproteins gp45 and gp90 (Montelaro *et al*., 1984). There is evidence of strain variation in the p26 amino acid sequence; however there is no evidence to indicate that this variation influences any of the serological diagnostic tests (Zhang *et al*., 1999).

- **Preparation of standard antiserum**

A known positive antiserum may be collected from a horse previously infected with EIAV. This serum should yield a single dense precipitation line that is specific for EIA, as demonstrated by a reaction of identity with a known standard serum. It is essential to balance the antigen and antibody concentrations in order to ensure the optimal sensitivity of the test. Reagent concentrations should be adjusted to form a narrow precipitation line approximately equidistant between the two wells containing antigen and serum.

- **Test procedure (Association Française de Normalisation [AFNOR], 2000; Coggins *et al*., 1973; Pearson & Coggins, 1979)**

i) Immunodiffusion reactions are carried out in a layer of agar in Petri dishes. For Petri dishes that are 100 mm in diameter, 15–17 ml of 1% Noble agar in 0.145 M borate buffer (9 g H_3BO_3, plus 2 g NaOH per litre), pH 8.6 (± 0.2) is used. Six wells are punched out of the agar surrounding a centre well of the same diameter. The wells are 5.3 mm in diameter and 2.4 mm apart. Each well must contain the same volume of reagent.

ii) The antigen is placed in the central well and the standard antiserum is placed in alternate exterior wells. Serum samples for testing are placed in the remaining three wells.

iii) The dishes are maintained at room temperature in a humid environment.

iv) After 24–48 hours the precipitation reactions are examined over a narrow beam of intense, oblique light and against a black background. The reference lines should be clearly visible at 24 hours, and at that time any test sera that are strongly positive may also have formed lines of identity with those between the standard reagents. A weakly positive reaction may take 48 hours to form and is indicated by a slight bending of the standard serum precipitation line between the antigen well and the test serum well. Sera with high precipitating antibody titres may form broader precipitin bands that tend to be diffuse. Such reactions can be confirmed as specific for EIA by dilution at 1/2 or 1/4 prior to retesting; these then give a more distinct line of identity. Sera devoid of EIA antibody will not form precipitation lines and will have no effect on the reaction lines of the standard reagents.

v) *Interpretation of the results:* Horses that are in the early stages of an infection may not give a positive serological reaction in an AGID test. Such animals should be bled again after 3–4 weeks. In order to make a diagnosis in a young foal, it may be necessary to determine the antibody status of the dam. If the mare passes EIA antibody to the foal through colostrum, then a period of 6 months or longer after birth must be allowed for the maternal antibody to wane. Sequential testing of the foal at monthly intervals may be useful to observe the decline in maternal antibody. To conclude that the foal is not infected, a negative result must be obtained (following an initial positive result) at least 2 months after separating the foal from contact with the EIA positive mare or any other positive horse. Alternatively PCR could be performed on the blood of the foal to determine the presence/absence of EIA proivirus.

b) Enzyme-linked immunosorbent assay

There are four ELISAs that are approved by the United States Department of Agriculture for the diagnosis of equine infectious anaemia and are available internationally; a competitive ELISA and three non-competitive ELISAs. The competitive ELISA and two non-competitive ELISAs detect antibody produced against the p26 core protein antigen. The third non-competitive ELISA incorporates both p26 core protein and gp45 (viral transmembrane protein) antigens. Typical ELISA protocols are used in all tests. If commercial ELISA materials are not available, a non-competitive ELISA using p26 antigen purified from cell culture material may be employed (Shane *et al*., 1984).

A positive test result by ELISA should be retested using the AGID test to confirm the diagnosis because some false-positive results have been noted with the ELISA. The results can also be confirmed by the immunoblot technique. A standard antiserum for immunodiffusion, which contains the minimum amount of antibody that should be detected by laboratories, is available from the OIE Reference Laboratories (see Table given in Part 4 of this *Terrestrial Manual*). Uniform methods for EIA control have been published (United States Department of Agriculture [USDA], 2002).

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

~~No biological products are available currently.~~

Inactivated and subunit EIAV vaccines were tested in different laboratories and proved to protect infections of homologous prototype strains only. An attenuated live vaccine, developed in the early 1970s, was extensively used in China (People's Rep. of) between 1975 and 1990 and was effective in controlling the prevalence of EIA. With low prevalence since 1990, the strategy for EIA control has shifted from vaccination to quarantine to avoid the interference of vaccine antibodies with diagnostic tests.

Although no safety concerns arose with the use of attenuated EIAV vaccine in China, it should be noted that, like other lentiviruses, EIAV is highly mutable and can integrate into host genomes. The use of a live EIAV vaccine needs to be very cautious and carefully evaluated.

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NB: There are OIE Reference Laboratories for Equine infectious anaemia:
(see Table in Part 4 of this *Terrestrial Manual* or consult the OIE web site for the most up-to-date list:
http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/ http://www.oie.int/).
Please contact the OIE Reference Laboratories for any further information on
diagnostic tests and reagents for Equine infectious anaemia

CHAPTER 2.5.10.

EQUINE VIRAL ARTERITIS

SUMMARY

Equine viral arteritis (*EVA*) *is a contagious viral disease of equids caused by equine arteritis virus* (*EAV*), *an RNA virus classified in the genus,* Arterivirus*, family* Arteriviridae*. ~~Only one major serotype of the virus has been identified so far.~~ Equine arteritis virus is found in horse populations in many countries world-wide. Although infrequently reported in the past, confirmed outbreaks of EVA appear to be on the increase.*

Description of the disease: *The majority of naturally acquired infections with EAV are subclinical. Where present, clinical signs of EVA can vary in range and severity. The disease is characterised principally by fever, depression, anorexia, dependent oedema, especially of the limbs, scrotum and prepuce in the stallion, conjunctivitis, an urticarial-type skin reaction, abortion and, rarely, a fulminating pneumonia, enteritis or pneumo-enteritis in young foals. Apart from mortality in young foals, the case-fatality rate in outbreaks of EVA is very low. Affected horses almost invariably make complete clinical recoveries. A long-term carrier state can occur in a variable percentage of infected stallions, but not in mares, geldings or sexually immature colts.*

Identification of the agent: *EVA cannot be differentiated clinically from a number of other respiratory and systemic equine diseases. Diagnosis of EAV infection is laboratory dependent and based on virus isolation, detection of nucleic acid or viral antigen, or demonstration of a specific antibody response. Detection and identification of EAV nucleic acid in suspect cases of the disease can be attempted using various reverse-transcription polymerase chain reaction* (*RT-PCR*) *assays. ~~Virus isolation should be attempted from appropriate clinical or post-mortem specimens in rabbit, equine, or monkey kidney cell culture.~~ The identity of isolates of EAV should be confirmed by RT-PCR assay, neutralisation test, or by immunocytochemical methods, namely indirect immunofluorescence or avidin–biotin–peroxidase techniques.*

~~Detection and identification of EAV nucleic acid in suspect cases of the disease can also be attempted using the RT-PCR assay and appropriate viral-specific RNA primers.~~

Where mortality is associated with a suspected outbreak of EVA, a wide range of tissues should be examined for histological evidence of panvasculitis that is especially pronounced in the small arteries throughout the body. The characteristic vascular lesions present in the mature animal are not a notable feature in EVA-related abortions, ~~In such cases, equine arteritis viral~~ diagnosis of which is based on virus isolation, viral nucleic acid detection by RT-PCR or demonstration of EAV antigens by immunohistochemical examination of placental and various fetal tissues.

Serological tests: *A variety of serological tests, including virus neutralisation* (*VN*), *complement fixation* (*CF*), *indirect fluorescent antibody, agar gel immunodiffusion, the enzyme-linked immunosorbent assay* (*ELISA*), *and the fluorescent microsphere immunoassay assay* (*MIA*) *have been used for the detection of antibody to EAV. The tests currently in widest use are the complement-enhanced VN test and the ELISA. The VN test is a very sensitive and highly specific assay of proven value in diagnosing acute infection and in seroprevalence studies. Several ELISAs have been developed. Although none ~~of which~~ have been as extensively validated as the VN test, ~~though~~ some ~~appear to~~ offer comparable specificity and close to equivalent sensitivity. The CF test is less sensitive than either VN test or ELISA ~~procedure~~, but it can be used for diagnosing recent infection.*

Requirements for vaccines ~~and diagnostic biologicals~~: *Two commercial tissue culture derived vaccines are currently available against EVA. One is a modified live virus* (*MLV*) *vaccine prepared from virus that has been attenuated for horses by multiple serial transfers in primary equine and*

rabbit kidney cells and in an equine dermal cell line ~~cultures~~. It has been ~~shown~~ confirmed to be safe and protective for stallions and nonpregnant mares. Vaccination of foals ~~under~~ less than 6 weeks of age and of pregnant mares in the final 2 months of gestation is ~~contraindicated~~ not recommended. There is no evidence of back reversion of the vaccine virus to virulence or of recombination with naturally occurring strains of EAV ~~the vaccine virus~~ following its use in the field ~~over more than 20 years~~. The second vaccine is an inactivated, adjuvanted product prepared from virus grown in equine cell culture that can be used in nonbreeding and breeding horses. In the absence of appropriate safety data, the vaccine is not currently recommended for use in pregnant mares.

A. INTRODUCTION

Definition of disease and aetiology: Equine viral arteritis (EVA) is a contagious viral disease of equids caused by equine arteritis virus (EAV), a positive-sense, single-stranded RNA virus, and the prototype member of the genus *Arterivirus,* family *Arteriviridae*, order *Nidovirales* (Cavanagh, 1997). Only one major serotype of the virus has been identified so far. Epizootic lymphangitis pinkeye, fièvre typhoide and rotlaufseuche are some of the descriptive terms used in the past to refer to a disease of close clinical resemblance to EVA. The natural host range of EAV would appear to be restricted to equids, although very limited evidence would suggest it may also include new world camelids, *viz*. alpacas and llamas (Weber *et al*., 2006). The virus does not present a human health hazard (Timoney & McCollum, 1993). ~~EAV is present in the horse population of many countries world-wide (Timoney & McCollum, 1993). There has been an increase in the incidence of EVA in recent years that has been linked to the greater frequency of movement of horses and use of transported semen (Balasuriya *et al*., 1998).~~

Description of disease: While the majority of cases of acute infection with EAV are subclinical, certain strains of the virus can cause disease of varying severity (Timoney & McCollum, 1993). Typical cases of EVA can present with all or any combination of the following clinical signs: fever, depression, anorexia, leukopenia, dependent oedema, especially of the limbs, scrotum and prepuce of the stallion, conjunctivitis, ocular discharge, supra or periorbital oedema, rhinitis, nasal discharge, a local or generalised urticarial skin reaction, a period of temporary subfertility in acutely affected stallions, abortion, stillbirths and, rarely, a fulminating pneumonia, enteritis or pneumo-enteritis in young foals. Regardless of the severity of clinical signs, affected horses almost invariably make complete recoveries. The case-fatality rate in outbreaks of EVA is very low; mortality is usually only seen in very young foals, particularly those congenitally infected with the virus (Timoney & McCollum, 1993; Vaala *et al*., 1992), and very rarely in otherwise healthy adult horses.

A variable percentage of acutely infected stallions later become long-term carriers in the reproductive tract and constant semen shedders of the virus (Timoney & McCollum, 1993). The carrier state, which has been shown to be androgen dependant, has been found in the stallion, but not in the mare, gelding or sexually immature colt (Timoney & McCollum, 1993). While temporary down-regulation of circulating testosterone levels using a GnRH antagonist or by immunisation with GnRH would appear to have expedited clearance of the carrier state in some stallions, the efficacy of either treatment strategy has yet to be fully established. Concern has been expressed that such a therapeutic approach could be used to deliberately mask existence of the carrier state.

~~EVA cannot be differentiated clinically from a number of other respiratory and systemic equine diseases, the most common of which are equine influenza, equine herpesvirus 1 and 4 infections, infection with equine rhinitis A and B viruses, equine adenoviruses and streptococcal infections, with particular reference to purpura haemorrhagica. The disease also has clinical similarities to equine infectious anaemia, African horse sickness fever, cases of Hendra virus infection, Getah virus infection and toxicosis caused by hoary alyssum (*Berteroa incana)* (Timoney & McCollum, 1993). After infection, EAV replicates in macrophages and circulating monocytes (Del Piero, 2000) and is shed in various secretions/excretions of acutely infected animals, in especially high concentration from the respiratory tract (McCollum *et al*., 1971).~~

~~A variable percentage of acutely infected stallions later become long-term carriers in the reproductive tract and constant semen shedders of the virus (Timoney & McCollum, 1993; 2000). The carrier state, which has been shown to be androgen dependant, has been found in the stallion, but not in the mare, gelding or sexually immature colt (Timoney & McCollum, 1993). Unequivocal evidence of the carrier state has only been found in stallions serologically positive for antibodies to the virus (Timoney & McCollum, 2000). While temporary down-regulation of circulating testosterone levels using a GnRH antagonist or by immunisation with GnRH would appear to have expedited clearance of the carrier state in some stallions, the efficacy of either treatment strategy has yet to be fully established. Concern has been expressed that such a therapeutic approach could be used to deliberately mask existence of the carrier state.~~

The gross and microscopic lesions described in fatal cases of EVA reflect the extensive vascular damage caused by the virus (Del Piero, 2000). EAV causes widespread vasculitis, primarily of the smaller arterioles and venules. This gives rise to oedema, congestion and haemorrhages, especially in the subcutis of the limbs and abdomen,

and excess peritoneal, pleural and pericardial fluid (Jones *et al*., 1957). Pulmonary oedema, emphysema and interstitial pneumonia, enteritis and splenic infarcts have been described in fatal cases of EVA in young foals (Del Piero, 2000). Gross lesions are usually absent in cases of abortion and microscopic changes, and if present, are most often seen in the placenta, liver, spleen and lungs of the fetus.

Factors of considered importance in the epidemiology of EVA are phenotypic variation among virus strains, modes of transmission during acute and chronic phases of infection, carrier state in the stallion, nature and duration of acquired immunity and changing trends in the horse industry. EAV is present in the horse population of many countries world-wide (Timoney & McCollum, 1993). There has been an increase in the incidence of EVA in recent years that has been linked to the greater frequency of movement of horses and use of transported semen (Balasuriya *et al*., 1998). Transmission of EAV can occur by respiratory, venereal and congenital routes. Respiratory spread is most important during the acute phase of the infection. EAV can also be transmitted venereally by the acutely infected stallion or mare and by the carrier stallion.

Current EVA control programmes are aimed at preventing the dissemination of EAV in breeding populations to minimise the risk of abortion outbreaks, deaths in young foals and establishment of the carrier state in colts and stallions (Timoney & McCollum, 1993). Such programmes are based on observance of sound management practices in conjunction with a targeted vaccination program of breeding stallions and sexually immature colts against the disease.

Differential diagnosis: EVA cannot be differentiated clinically from a number of other respiratory and systemic equine diseases, the most common of which are equine influenza, equine herpesvirus 1 and 4 infections, infection with equine rhinitis A and B viruses, equine adenoviruses and streptococcal infections, with particular reference to purpura haemorrhagica. The disease also has clinical similarities to equine infectious anaemia, equine encephalosis virus infection, African horse sickness fever, cases of Hendra virus infection, Getah virus infection and toxicosis caused by hoary alyssum (*Berteroa incana*) (Timoney & McCollum, 1993).

B. DIAGNOSTIC TECHNIQUES

Table 1. *Test methods available and their purpose*

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection	Efficiency of eradication policies	Confirmation of clinical cases	Prevalence of infection - surveillance	Immune status in individual animals or populations post-vaccination
Virus isolation	–	+++	–	+++	–	–
Agar gel immunodiffusion	–	–	–	–	–	–
Complement fixation	–	–	–	+++	–	–
Enzyme-linked immunosorbent assay	+	++	+	++	+++	+
Polymerase chain reaction	–	+++	–	+++	–	–
Virus neutralisation	+	+++	+	+++	+++	+++

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose.
Although not all of the tests listed as category +++ or ++ have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

Detection and identification of EAV from appropriate clinical and tissue samples can be accomplished by virus isolation in cell culture and by detection of viral nucleic acid using a range of reverse-transcription polymerase

chain reaction (RT-PCR) assays. Both diagnostic approaches are appropriate for confirmation of clinical cases of EVA as well as establishing individual animal freedom from EAV infection. In the latter context, virus isolation and RT-PCR assays have been used in surveillance studies and in enabling animal movement to take place. Antigen detection through the use of various immunolabelling techniques also has diagnostic application when examining tissues from suspect cases of EVA abortion, death in young foals or older horses.

Isolation of EAV can be attempted in a limited number of cell lines of which the RK-13 rabbit kidney cell line (ATCC CCL37, or RK13-KY [1]) has proven to be optimal especially when testing stallion semen. Several comprehensive comparison studies have shown virus isolation to be of equivalent sensitivity to RT-PCR for the detection of EAV in clinical and morbid material. Although many isolations of the virus are made in initial passage in cell culture, virus isolation is not a rapid diagnostic test in contrast to certain RT-PCR assays that can be completed in the same day.

A wide variety of RT-PCR assays (single step, nested, real-time) have been developed for EAV detection. Regrettably, very few have been adequately validated and compared with virus isolation for sensitivity and specificity. It is important to emphasise that the choice of reagent kits for both nucleic acid and extraction and amplification in the real-time RT-PCR assay can have a major influence on the overall diagnostic sensitivity and robustness of the assay (Miszczak *et al.*, 2011).

Immunohistochemical testing for EAV antigen in frozen or fixed tissue sections is best accomplished using a monospecific polyclonal serum against the virus or a monoclonal antibody (MAb) directed against the highly conserved nucleocapsid (N) viral protein.

Of the serological tests evaluated for the detection of antibodies to EAV, the complement-enhanced virus neutralisation (VN) test has been proven the most reliable for the diagnosis of acute EAV infection and for serosurveillance studies. Of the numerous enzyme-linked immunosorbent assays (ELISAs) that have been developed, a few offer comparable but not identical sensitivity and specificity to the VN test. A benefit of an EAV ELISA is that it can provide a same-day test result compared with the VN test, which is a 72-hour test. None of the available tests can reliably differentiate antibody titres resulting from natural infection from those due to vaccination.

1. Identification of the agent

a) ~~Virus isolation~~ *In-vitro* culture

In the event of a suspect outbreak of EVA, or when endeavouring to confirm a case of subclinical EAV infection, virus isolation should be attempted preferably from nasopharyngeal or deep nasal swabs, conjunctival swabs, unclotted blood samples, and semen from stallions considered ~~to be possible~~ putative carriers of the virus (Timoney & McCollum, 1993). To optimise the chances of virus isolation during an outbreak, relevant specimens should be obtained as soon as possible after the onset of fever in affected horses. In attempting virus isolation from peripheral mononuclear cells (PBMCs), blood should be collected in citrate on ethylenediaminetetraacetic acid (EDTA) anticoagulant. As heparin can inhibit the growth of EAV in rabbit kidney cells (RK-13 cell line), its use as an anticoagulant is contra-indicated as it may interfere with isolation of the virus from whole blood. ~~Acid citrate dextrose or ethylenediaminetetraacetic acid (EDTA) are the anticoagulants of choice to use in obtaining unclotted blood samples.~~ Where EVA is suspected in cases of mortality in young foals or older animals, virus isolation can be attempted from a variety of tissues, especially the lymphatic glands associated with the alimentary tract and related organs, and also the lungs, liver and spleen (McCollum *et al.*, 1971). In outbreaks of EVA-related abortion and/or cases of stillborn foals, placental and fetal fluids and a wide range of placental, lymphoreticular and other fetal tissues (especially lung) can be productive sources of virus (Timoney & McCollum, 1993).

Swabs for attempted isolation should be immersed in a suitable viral transport medium and these, together with any fluids or tissues collected for virus isolation and/or RT-PCR testing should be shipped either refrigerated or frozen in an insulated container to the laboratory, ~~preferably using an overnight delivery service~~ ideally within 24 hours. If swabs are intended for direct examination by RT-PCR, ~~they should not be made of wood, as preservatives therein may interfere with the PCR reaction~~ the swab shaft should not be made of wood, which might contain substances such as preservatives that could interfere with the PCR reaction. Unclotted blood samples must be transported refrigerated but not frozen. Where possible, specimens should be submitted to a laboratory with established competency in testing for this infection.

Nasopharyngeal swabs in transport medium are processed by transferring each into the barrel of a 10 ml syringe, the syringe plunger inserted and whatever fluid can be extracted is collected into a sterile tube. An

1 Available from the OIE Reference Laboratory for Equine viral arteritis in the United States of America (USA) (consult the OIE web site for full contact details: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/)

aliquot of the fluid is passed through a prefilter and then filtered through a 0.45 µm membrane syringe fitter and collected aseptically for subsequent inoculation into cell culture.

Buffy coats can be harvested from unclotted blood by centrifugation at 600 ***g*** for 15 minutes, and the buffy coat taken off after the plasma has been carefully removed. The buffy coat is then layered onto a PBMC separating solution, Ficoll 1.077, and centrifuged at 400 ***g*** for 20 minutes. The PBMC interface (without most granulocytes) is washed twice in phosphate buffered saline (300 ***g*** for 10 minutes) and resuspended in 1 ml of Eagle's minimal essential medium (MEM) containing 2% FCS. A 0.5 ml volume of the rinsed cell suspension is added to monolayers of RK-13 cells in 25 cm^2 flasks or multiwall plates to which maintenance medium is added.

Although reportedly not always successful in natural cases of EAV infection (Timoney & McCollum, 1993), virus isolation should be attempted from clinical specimens or necropsy tissues using rabbit, equine or monkey kidney cell culture (Timoney *et al*., 2004; Timoney & McCollum, 1993). Selected cell lines, e.g. RK-13 (ATCC CCL-37), LLC-MK_2 (ATCC CCL-7), and primary horse or rabbit kidney cell culture can be used, with RK-13 cells being the cell system of choice (Timoney *et al*., 2004). Experience over the years has shown that primary isolation of EAV from semen can present more difficulty than from other clinical specimens or from infected tissues unless an appropriate cell culture system is used. Several factors have been shown to influence primary isolation of EAV from semen in RK-13 cells. Higher isolation rates have been obtained using 3- to 5-day-old confluent monolayers, a large inoculum size in relation to the cell surface area in the inoculated flasks or multiwell plates, and most importantly, the incorporation of carboxymethyl cellulose (medium viscosity, 400–800 cps) in the overlay medium. It should be noted that most RK-13 cells, including ATCC CCL-37, are contaminated with bovine viral diarrhoea virus, the presence of which appears to enhance sensitivity of this cell system for the primary isolation of EAV, especially from semen. ~~There is considerable evidence to indicate that primary~~ In the case of specimens of low viral infectivity, isolation rates of EAV may be increased by using RK-13 cells of high passage history[2] (Timoney *et al*., 2004).

Inoculated cultures are examined daily for the appearance of viral cytopathic effect (CPE), which is usually evident within 2–6 days. In the absence of visible CPE, culture supernatants should be subinoculated on to confluent cell monolayers after 4–7 days. While the vast majority of isolations of EAV are made on the first passage in cell culture, a small minority only become evident on the second or subsequent passages *in vitro* (Timoney & McCollum, 1993~~; 2000~~). The identity of isolates of EAV can be confirmed by standard or real-time RT-PCR assays (Balasuriya *et al*., 1998), in a one-way neutralisation test, or by an immunocytochemical method (Little *et al*., 1995), indirect immunofluorescence (Crawford & Henson, 1973) or avidin–biotin–peroxidase (ABC) technique (Little *et al*., 1995). A polyclonal rabbit antiserum has been used to identify EAV in infected cell cultures. Mouse monoclonal antibodies (MAbs) to the nucleocapsid protein (N) ~~(Balasuriya *et al*., 1998)~~ and major envelope glycoprotein (GP5) of EAV ~~(Balasuriya *et al*., 1998)~~ as well as a monospecific rabbit antiserum to the unglycosylated envelope protein (M) (Balasuriya *et al*., 1998) have also been developed and these can detect various strains of the virus in RK-13 cells as early as 12–24 hours after infection (Balasuriya *et al*., 1998; Little *et al*., 1995).

- **Virus isolation from semen (a prescribed test for international trade)**

There is considerable evidence that short- and long-term carrier stallions shed EAV constantly in the semen, but not in respiratory secretions or urine; nor has it been demonstrated in the buffy coat (peripheral blood mononuclear cells) of such animals (Timoney *et al*., 1987; Timoney & McCollum, 1993~~; 2000~~). Stallions should first be blood tested using the VN test or an appropriately validated ELISA or other serological test procedure. Virus isolation should be attempted from the semen of stallions serologically positive for antibodies to EAV e.g.VN titre ≥1/4, that do not have a certified history of vaccination against EVA, also with confirmation that they were serologically negative (VN titre <1/4) at time of initial vaccination. Virus isolation is also indicated in the case of shipped semen where the serological status and possible vaccination history of the donor stallion is not available. It is recommended that virus isolation from semen be attempted from two samples, which can be collected on the same day, on consecutive days, or after an interval of several days or weeks. There is no evidence that the outcome of attempted virus isolation from particular stallions is influenced by the frequency of sampling, the interval between collections, or time of the year. Isolation of EAV should be carried out preferably on portion of an entire ejaculate collected using an artificial vagina or a condom and a teaser or phantom mare. When it is not possible to obtain semen by this means, a less preferable alternative is to collect a dismount sample at the time of breeding. Care should be taken to ensure that no antiseptics/disinfectants are used in the cleansing of the external genitalia of the stallion prior to collection. Samples should contain the sperm-rich fraction of the ejaculate with which EAV is associated as the virus is not present in the pre-sperm fraction of semen (Timoney *et al*., 1987; Timoney & McCollum, 1993~~; 2000~~). Immediately following collection, the semen should be refrigerated on crushed ice or on freezer

2 Such a line (RK-13-KY) is available from the OIE Reference Laboratory for Equine viral arteritis in the USA (consult the OIE web site for full contact details: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/)).

packs for transport to the laboratory as soon as possible. Where there is likely to be a delay in submitting a specimen for testing, the semen can be frozen at or below –20°C for a short period before being dispatched to the laboratory. Freezing a sample does not appear to interfere with isolation of EAV from semen. In situations where it is not feasible to determine the carrier status of a stallion by virus isolation or RT-PCR assay, the stallion can be test bred to two seronegative mares, which are checked for seroconversion to the virus up to 28 days after breeding (Timoney & McCollum, 1993).

It is not possible to reliably determine the carrier status of stallions treated with GnRH antagonist or immunised with GnRH to modify reproductive activity or behaviour, as this may also temporarily interrupt EAV shedding.

- **Test procedure**

i) On receipt in the laboratory, it should be noted whether a semen sample is frozen, chilled or at ambient temperature. Every sample should be checked to ensure that it contains the sperm-rich fraction of the ejaculate. ~~This can be established by microscopic examination of a wet mount preparation of a sample~~ Some stallions can produce large volumes of seminal plasma prior to ejaculating the sperm-rich and gel fractions of semen. Frequently, this pre-sperm fraction contains very few sperm and can be EAV negative even though the stallion is a carrier of EAV (Timoney *et al*., 1987). To optimise detection of virus, 50 µl of each semen sample should be transferred onto a glass slide, covered with a cover-slip and examined microscopically at a magnification of 100× to assess its sperm content. Ejaculates with less than an average of five sperm per ten fields examined should be considered of questionable diagnostic value. It is worth noting however, that the occasional oligospermic stallion can be EAV positive even with a low sperm count. If virus negative on the other hand, the test report on such a stallion should include the qualifier that freedom from EAV cannot be guaranteed based on the low sperm numbers in the sample submitted. Additionally, specimens of ejaculate should be visually inspected and recorded for colour and presence of gross particulate contamination. If a semen specimen is contaminated with blood, which can result from trauma to the external genitalia of the stallion at time of collection, a repeat sample should be requested as testing such a specimen from a serologically positive stallion may compromise the reliability of the virus isolation result due to the EAV antibodies in the serum. Very infrequently, an ejaculate may have a yellowish tinge due to contamination with urine. Such samples may be positive for equine rhinitis A virus.

ii) Although no longer considered an essential step, pretreatment of semen before inoculation into cell culture by short-term sonication (three 15-second cycles); facilitates effective mixing and dispersion of a sample.

iii) After removal of culture medium, 3- to 5-day-old confluent monolayer cultures of RK-13 cells, either in 25 cm^2 tissue culture flasks or multiwell plates, are inoculated with serial decimal dilutions (from 10^{-1} to 10^{-3}) of seminal plasma in tissue culture maintenance medium containing 2% fetal bovine serum and antibiotics. An inoculum of 1 ml per 25 cm^2 flask is used and no fewer than two flasks per dilution of seminal plasma are inoculated. Inoculum size and number of wells inoculated per dilution of a specimen should be pro-rated where multiwell plates are used. Appropriate dilutions of a virus positive control semen sample or virus control of known titre diluted in culture medium should be included in each test.

iv) The flasks are closed, lids replaced on multiwell plates and inoculated cultures gently rotated to disperse the inoculum over the cell monolayers.

v) Inoculated cultures are then incubated for 1 hour at 37°C either in an aerobic incubator or an incubator containing a humidified atmosphere of 5% CO2 in air, depending on whether flasks or multiwell plates are used.

vi) Without removing any of the inoculum or washing the cell monolayers, the latter are overlaid with 0.75% carboxymethyl cellulose containing medium with antibiotics.

vii) The flasks or plates are reincubated at 37°C and checked microscopically for viral CPE, which is usually evident within 2–6 days.

viii) In the absence of visible CPE, culture supernatants are subinoculated onto 3–5 day-old confluent cell monolayer cultures of RK-13 cells after 5–7 days. After removal of the overlay medium, monolayers are stained with 0.1% formalin-buffered crystal violet solution.

The identity of any virus isolates should be confirmed by standard or real-time RT-PCR (Balasuriya *et al*., 1998; Gilbert *et al*., 1997; Balasuriya *et al*., 2002; Lu *et al*., 2007; Westcott *et al*., 2003; Miszczak *et al*., 2011) by VN, immunofluorescence (Crawford & Henson, 1973) or ABC technique, using a monospecific antiserum to EAV or MAbs to the structural proteins, N or GP5 of the virus (Balasuriya *et al*., 1998; Del Piero, 2000; Little *et al*., 1995).

In the one-way neutralisation test, serial decimal dilutions of the virus isolate are tested against a neutralising MAb or monospecific antiserum prepared against the prototype Bucyrus strain of EAV (ATCC VR 796) and also a serum negative for neutralising antibodies to the virus. Corresponding titrations of the prototype Bucyrus virus with the same reference antibody reagents are included as test controls. The test is performed in either 25 cm^2 tissue culture flasks or multiwell plates. Appropriate quantities of the known EAV positive and negative antibody reagents are inactivated for 30 minutes in a water bath at 56°C and diluted 1/4 in phosphate buffered saline, pH 7.2; then 0.3 ml of diluted antibody reagent is dispensed into five tubes for each ~~virus~~ isolate to be tested. Serial decimal dilutions (from 10^{-1} to 10^{-5}) of each virus isolate are made in Eagle's MEM containing 10% fetal bovine serum, antibiotics and 10% freshly diluted guinea-pig complement. Then, 0.3 ml of each virus dilution is added to the tubes containing positive and negative antibody reagents. The tubes are shaken and the virus/antibody mixtures are incubated for 1 hour at 37°C. The mixtures are then inoculated onto 3–5-day-old confluent monolayer cultures of RK-13 cells, either in 25 cm^2 flasks or multiwell plates, using two flasks or wells per virus dilution. Each flask is inoculated with 0.25 ml of virus/antibody mixture; the inoculum size is pro-rated where multiwell plates are used. Inoculated flasks or plates are incubated for 2 hours at 37°C, gently rocking after 1 hour to disperse the inoculum over the cell monolayers. Without removing any of the inoculum or washing the cell monolayers, the latter are overlaid with 0.75% carboxymethyl cellulose containing medium and incubated for 4–5 days at 37°C, either in an aerobic incubator or an incubator containing a humidified atmosphere of 5% CO_2 in air. After removal of the medium, monolayers are stained with 0.1% formalin-buffered crystal violet solution. Plaques are counted and the virus infectivity titre is determined both in the presence and absence of EAV antibodies using the Spearman–Kärber method. Confirmation of the identity of an isolate is based on a reduction in plaque count of at least 10^2 logs of virus in the presence of antibody positive serum against the Bucyrus strain of EAV.

The vast majority of EAV isolates from carrier stallions are made in the first passage in cell culture using the described test procedure (Timoney & McCollum, 1993~~; 2000~~). The occurrence of nonviral cytotoxicity or bacterial contamination of specimens is not ~~considered~~ a significant problem~~s~~ when attempting isolation of this virus from stallion semen. Nonviral cytotoxicity, if observed, usually affects monolayers inoculated with the 10^{-1} and, much less frequently, the 10^{-2} dilution of seminal plasma. Treatment of seminal plasma with polyethylene glycol (Mol. wt 6000) prior to inoculation has been used with some success in overcoming this problem (Fukunaga *et al*., 2000). The method described involves the addition of polyethylene glycol to the 10^{-1} to 10^{-3} dilutions of seminal plasma to give a final concentration of 10% in each dilution. The mixtures are held overnight at 4°C with gentle stirring, after which they are centrifuged at 2000 ***g*** for 30 minutes and the supernatants are discarded. The precipitates are suspended in cell culture maintenance medium to one-tenth the volume of the original dilutions and the mixtures are homogenised. They are then centrifuged at 2000 ***g*** for 30 minutes and the supernatants are taken off and used for inoculation. There is no evidence to indicate that pretreatment of seminal plasma in this manner reduces sensitivity of the virus isolation procedure (Fukunaga *et al*., 2000). Where bacterial contamination of a sample is a problem, it is preferable to request a repeat semen collection from the individual stallion. If this is not possible, an attempt can be made to control the contamination by pre-treatment of the sample with antibiotic containing viral transport medium, holding overnight at 4°C followed by ultracentrifugation and resuspension of the pellet before diluting and inoculating the specimen into cell culture.

~~The presence of anti-EAV antibody activity in the seminal plasma of certain virus-shedding stallions has not been found to prevent detection of the carrier state in these animals.~~

There have been two reports of failure to isolate EAV from individual stallions whose semen was positive for viral nucleic acid on RT-PCR assay. In one case at least, failure to detect infectious virus may well have been the result of a very high level of neutralising activity in the seminal plasma of the stallion, emphasising the value of RT-PCR as an adjunct to virus isolation for detection of EAV.

b) Antigen detection

Where mortality is associated with a suspected outbreak of EVA, a wide range of tissues should be examined for histological evidence of panvasculitis that is especially pronounced in the small arteries and venules throughout the body, particularly in the caecum, colon, spleen, associated lymphatic glands and adrenal cortex (Crawford & Henson, 1973; Del Piero, 2000; Jones *et al*., 1957). The presence of a disseminated necrotising arteritis involving endothelial and medial cells of affected vessels is considered a pathognomonic feature of EVA. The characteristic vascular lesions present in the mature animal are not, however, a prominent a feature in many cases of EAV-related abortion.

EAV antigen can be identified in various tissues of EVA-affected animals either in the presence or absence of lesions (Del Piero, 2000). Antigen has been demonstrated in lung, heart, liver and spleen and the placenta of aborted fetuses (Del Piero, 2000). Immunohistochemical examination of biopsied skin specimens has also been investigated as a means of confirming acute EAV infection. Though of some value, it is not entirely reliable for the diagnosis of the disease. Viral antigen can be detected within the cytoplasm of

infected cells by immunofluorescence using conjugated equine polyclonal anti-EAV serum (Crawford & Henson, 1973), or by the ABC technique using mouse MAbs to the GP5 or N proteins of the virus (Del Piero, 2000).

c) ~~Nucleic acid recognition~~ Molecular methods

The standard two-step RT-PCR, single-step RT-PCR, RT-nested PCR, and real-time RT-PCR (rRT-PCR) assays have become widely accepted as an alternative or adjunct to virus isolation in cell culture for the detection of EAV in diagnostic materials. The RT-PCR-based assays provide a means of identifying virus-specific RNA in clinical specimens, namely nasopharyngeal or nasal swab filtrates, buffy coats, raw and extended semen and urine, and in post-mortem tissue samples (Balasuriya *et al*., 2002; Gilbert *et al*., 1997; Lu *et al.,* 2007; Miszczak *et al*., 2011; Westcott *et al*., 2003;). Standard ~~two-step RT-PCR~~, single-step RT-PCR, RT-nested PCR (RT-nPCR), and one tube TaqMan® rRT-PCR assays have been developed and evaluated for the detection of various strains of the virus in tissue culture fluid, semen and nasal secretions (Balasuriya *et al*., 2002; Gilbert *et al*., 1997; Lu *et al*., 2007; Miszczak *et al*., 2011; Westcott *et al*., 2003;). These assays targeted six different open reading frames (ORFs) in the EAV genome (ORFs 1b, 3–7). However, there is considerable variation in the sensitivity and specificity among RT-PCR assays incorporating different primer pairs targeting various ORFs. Results comparable to virus isolation have been obtained with some but not all standard single-step RT-PCR, two-step RT-PCR, RT-nPCR or one tube TaqMan® rRT-PCR assays (Balasuriya *et al*., 2002; Gilbert *et al*., 1997; Lu *et al*., 2007; Miszczak *et al*., 2011). Compared with traditional virus isolation, these RT-PCR-based assays are frequently more sensitive ~~less expensive~~ and considerably more rapid to perform, the majority taking less than 24 hours to complete. In addition, RT-PCR assays have the advantage of not requiring viable virus for performance of the test. The one-tube rRT-PCR assay for EAV provides a simple, rapid and reliable method for the detection and identification of viral nucleic acid in equine semen and tissue culture fluid (Balasuriya *et al*., 2002; Lu *et al*., 2007; Miszczak *et al*., 2011). However, there is evidence to indicate that the choice of commercial kit used for nucleic acid extraction and also for amplification can have a major influence on the overall diagnostic sensitivity and robustness of the assay Miszczak *et al*., 2011). This was demonstrated using a magnetic-bead-based nucleic acid extraction method in combination with specific commercial RT-PCR kits. The one tube rRT-PCR has the following important advantages over the standard two-step RT-PCR: 1) eliminating the possibility of cross contamination between samples with previously amplified products as the sample tube is never opened; and 2) reducing the chance of false-positive reactions where the rRT-PCR product is detected with a sequence-specific probe. Because of the high sensitivity of the RT-PCR assay, however, and in the absence of appropriate safeguards in the laboratory, there is the potential for cross-contamination between samples, giving rise to false-positive results. For example, the RT-nPCR assay, while it provides enhanced sensitivity for the detection of EAV, it also increases the likelihood of false-positive results. The risk of cross-contamination is greater using the RT-nPCR assay because of the second PCR amplification step involving the product from the first RT-PCR reaction. To minimise the risk of cross-contamination, considerable care needs to be taken, especially during the steps of RNA extraction and reaction setup. Relevant EAV positive and negative template controls and, where appropriate, nucleic acid extracted from the tissue culture fluid of uninfected cells, need to be included in each RT-PCR assay. Thus, in ~~many~~ most circumstances, use of the single-step RT-PCR or one tube rRT-PCR assay will largely circumvent the problems associated with cross contamination.

Primer selection is critical to the sensitivity of the RT-PCR assay with primers (and probe in the case of the rRT-PCR assay) preferably designed from the most conserved region(s) of the EAV genome. Comparative nucleotide sequence analysis has shown that ORF 1b (encodes the viral polymerase), ORF 6 (M protein) and 7 (N protein) are more conserved than the other ORFs among EAV strains so far analysed from North America and Europe (Balasuriya *et al*., 2002; Lu *et al*., 2007; Miszczak *et al*., 2011; Westcott *et al*., 2003). The most conserved gene among different strains of EAV is ORF7 and primers specific for ORF7 (and probe for rRT-PCR) have detected a diversity of strains of the virus of European and North American origin (Balasuriya *et al*., 2002; Lu *et al*., 2007). Furthermore, the use of multiple primer pairs specific for different ORFs 1b ([forward: 5'-GAT-GTC-TAT-GCT-CCA-TCA-TT-3' and reverse: 5'-GGC-GTA-GGC-TCC-AAT-TGA-A-3']) and/or [forward: 5'-CCT-GAG-ACA-CTG-AGT-CGC-GT-3' and reverse 5'-CCT-GAT-GCC-ACA-TGG-AAT-GA-3']) (Gilbert *et al*., 1997), ORF 6 ([forward: 5'-CTG-AGG-TAT-GGG-AGC-CAT-AG-3' and reverse: 5'-GCA-GCC-AAA-AGC-ACA-AAA-GC-3']) and ORF 7 ([forward 5'-ATG-GCG-TCA-AGA-CGA-TCA-CG-3' and reverse 5'-AGA-ATA-TCC-ACG-TCT-TAC-GGC-3']) markedly increases the likelihood of detecting North American and European strains of EAV in the RT-PCR assay. The two primer pairs specific for ORF 1b are suitable for use in a rRT-PCR assay (Gilbert *et al*., 1997). While the RT-PCR has been found to be highly sensitive for viral nucleic acid detection in raw semen, there is evidence that it is not of equivalent reliability when testing ~~extended or~~ cryopreserved semen of very low virus infectivity (Zhang *et al*., 2004).

In addition to the foregoing RT-PCR assays, 2 TaqMan® fluorogenic probe-based one-tube rRT-PCR assays have been described for the detection of EAV nucleic acid (Balasuriya *et al*., 2002); primers ([forward: 5'-GGC-GAC-AGC-CTA-CAA-GCT-ACA-3', reverse: 5'-CGG-CAT-CTG-CAG-TGA-GTG-A-3'] and probe [5'FAM-TTG-CGG-ACC-CGC-ATC-TGA-CCA-A-TAMRA-3'] and (Westcott *et al*., 2003); primers [forward: 5'-

GTA-CAC-CGC-AGT-TGG-TAA-CA-3', reverse: 5'-ACT-TCA-ACA-TGA-CGC-CAC-AC-3'] and probe [5'FAM-TGG-TTC-ACT-CAC-TGC-AGA-TGC-CGG-TAMRA-3']). It should be noted, however, that genomic variation among field isolates of EAV could reduce the sensitivity of both RT-PCR and rRT-PCR assays, even when the primers and probe are based on the most conserved region of the EAV genome (ORF 7 [Lu *et al*., 2007]). Phylogenetic studies of strains of EAV from certain regions/countries have confirmed the existence of clusters of isolates more closely related to one another than to virus strains of disparate geographic backgrounds (Mankoc *et al*., 2007). Under such circumstances, validated primers besides those already recommended may be more suitable for detection of these genomically distinct strains of EAV.

In the absence of ~~general~~ widespread agreement on a ~~complete consensus or~~ universal primer set for EAV, and as no RT-PCR assay can determine the actual infectivity of a sample, there is a value to performing virus isolation in conjunction with RT-PCR or rRT-PCR for the identification of virus in clinical or post-mortem specimens and where indicated, genomic and phenotypic analysis of viral isolates.

Strains of EAV isolated from different regions of the world have been classified into different phylogenetic groups by sequence analysis of the GP3, GP5 and M envelope protein genes (ORFs 3, 5 and 6 respectively) and the nucleocapsid (N) protein gene (ORF 7 [Balasuriya *et al*., 1998; ~~Stadejek *et al*., 1999~~ Zhang *et al*., 2010]). The GP5 gene has been found to be most useful and reliable for this purpose. The relationships between strains demonstrated by nucleotide sequencing are a useful molecular epidemiological tool for tracing the origin of outbreaks of EVA (Balasuriya *et al*., 1998; ~~Balasuriya *et al*., 2004~~ Zhang *et al*., 2010).

~~c) Histopathological and immunohistochemical methods~~

~~Where mortality is associated with a suspected outbreak of EVA, a wide range of tissues should be examined for histological evidence of panvasculitis that is especially pronounced in the small arteries throughout the body, particularly in the caecum, colon, spleen, associated lymphatic glands and adrenal cortex (Crawford & Henson, 1973; Del Piero, 2000; Jones et al., 1957). The presence of a disseminated necrotising arteritis involving endothelial and medial cells of affected vessels is considered to be pathognomonic of EVA. The characteristic vascular lesions present in the mature animal are not, however, a prominent a feature in many cases of EAV related abortion.~~

~~EAV antigen can be identified in various tissues of EVA affected animals either in the presence or absence of lesions (Del Piero, 2000). Antigen has been demonstrated in lung, heart, liver and spleen and the placenta of aborted fetuses (Del Piero, 2000). Immunohistochemical examination of biopsied skin specimens has also been investigated as a means of confirming acute EAV infection. Though of some value, it is not entirely reliable for the diagnosis of the disease. Viral antigen can be detected within the cytoplasm of infected cells by immunofluorescence using conjugated equine polyclonal anti-EAV serum (Crawford & Henson, 1973), or by the ABC technique using mouse MAbs to the GP5 (Glaser et al., 1995) or N proteins of the virus (Del Piero, 2000).~~

2. Serological tests

A variety of serological tests have been investigated for their ability to detect antibodies to EAV. These include the neutralisation (microneutralisation [Senne *et al*., 1985] and plaque reduction [McCollum, 1970]), the complement fixation (CF) test (Fukunaga & McCollum, 1977), the indirect fluorescent antibody test (Crawford & Henson, 1973), the agar gel immunodiffusion (Crawford & Henson, 1973), the ELISA (Cho *et al*., 2000; Hedges *et al*., 1998; Kondo *et al*., 1998; Nugent *et al*., 2000) and the fluorescent microsphere immunoassay (MIA) (Go *et al.,* 2008~~; pers. comm.) have been used to detect antibody to EAV~~.

~~The test currently in widest international use to diagnose infection, carry out seroprevalence studies, and test horses for export, is a microneutralisation test in the presence of complement. It has also been used to screen fetal heart blood for the retrospective diagnosis of cases of EVA related abortion. Apart from the VN test, the CF test has been used for diagnosing recent EAV infection as complement-fixing antibodies are relatively short-lived in duration (Fukunaga & McCollum, 1977). In contrast, neutralising antibody titres to EAV frequently persist for several years after natural infection (Timoney & McCollum, 1993). Although a number of ELISAs have been developed (Cho *et al*., 2000; Hedges *et al*., 1998; Kondo *et al*., 1998; Nugent *et al*., 2000), none has as yet been as extensively validated as the VN, though some appear to offer nearly comparable sensitivity and specificity (Hedges *et al*., 1998; Nugent *et al*., 2000). Unlike the VN test, a positive reaction in the ELISA is not necessarily reflective of the protective immune status of an individual horse to EAV as both non-neutralising and neutralising antibodies are involved.~~

Interestingly, only one major serotype of EAV represented by the prototype Bucyrus strain (ATCC VR 796) has been recognised so far (McCollum, 1970; Timoney & McCollum, 1993). Antiserum to unpurified EAV has been prepared in horses and in rabbits using conventional immunisation protocols. Mouse MAbs and monospecific rabbit antibodies have also been developed to the nucleocapsid protein (N) major envelope glycoprotein (GP5), and unglycosylated envelope protein (M) of EAV (Balasuriya *et al*., 1997~~; Glaser *et al*., 1995~~).

~~OIE Standard Sera for EAV are available [3] and these can facilitate international standardisation of the microneutralisation test and ELISA.~~

~~Only one major serotype of EAV has been recognised so far (McCollum, 1970; Timoney & McCollum, 1993). This is represented by the prototype Bucyrus strain (ATCC VR 796). The reference virus used in the EAV VN test is the CVL Bucyrus (Weybridge) strain. Virus stock is grown in the RK-13 cell line, clarified of cellular debris by low-speed centrifugation and stored in aliquots at –70°C.~~

Neutralisation test: The complement-enhanced microneutralisation is currently the test in widest international use to diagnose EAV infection, carry out seroprevalence studies, and test horses for movement. It has also been used to screen fetal heart blood as a means of retrospectively diagnosing cases of EVA-related abortion. Neutralising antibodies to EAV persist for several years after natural infection or vaccination with the modified live vaccine against EVA (Timoney & McCollum, 1993).

a) Virus neutralisation test (a prescribed test for international trade)

The VN test is used for diagnostic purposes to confirm infection in suspect cases/outbreaks of EVA and to screen horses e.g. stallions, for evidence of EAV infection ~~and to determine whether there is a need to attempt virus detection in semen using cell culture or RT-PCR assay. It is also used for diagnostic purposes to confirm infection in suspect cases of EVA~~. The test procedure currently in widest use is that developed by the National Veterinary Service Laboratories of the United States Department of Agriculture (Senne *et al*., 1985). It is important to obtain a sterile blood sample as bacterial contamination of serum can interfere with the test result. It is recommended that the test be carried out in RK-13 cells using the approved CVL-Bucyrus (Weybridge) strain of EAV as reference virus[4] (Edwards *et al*., 1999). Although originally derived from the prototype Bucyrus virus, the passage history of the CVL (Weybridge) strain is not fully documented. Stocks of the reference virus are grown in the RK-13 cell line, clarified of cellular debris by low-speed centrifugation and stored in aliquots at –70°C. Several frozen aliquots are thawed and the infectivity of the stock virus is determined by titration in RK-13 cells. The sensitivity of the VN test for detection of antibodies to EAV can be significantly influenced by several factors, especially the source and passage history of the strain of virus used (Edwards *et al*., 1999). The CVL-Bucyrus (Weybridge) strain and the highly attenuated MLV vaccine strain of EAV are of comparable sensitivity for detecting low-titred positive sera, especially from EVA-vaccinated horses. Efforts are continuing to bring about greater uniformity in the testing protocol and serological results among laboratories providing the VN or other comparable serological assays for this infection. OIE Approved Standard Sera for EAV are available [5] and these can facilitate international standardisation of the microneutralisation test and ELISA.

- **Test procedure**

i) Sera are inactivated for 30 minutes in a water bath at 56°C (control sera, only once).

ii) Serial twofold dilutions of the inactivated test sera in serum-free cell culture medium (25 µl volumes) are made in a 96-well, flat-bottomed, cell-culture grade microtitre plate starting at a 1/2 serum dilution and using duplicate rows of wells for each serum to be tested. Most sera are screened initially at a 1/4 and 1/8 serum dilution (i.e. final serum dilution after addition of an equal volume of the appropriate dilution of stock virus to each well). Positive samples at the 1/8 dilution can, if desired, be retested and titrated out for end-point determination. Individual serum controls, together with negative and known low- and high-titred positive control sera must also be included in each test.

iii) A dilution of stock virus made up to contain from 100 to 300 $TCID_{50}$ (50% tissue culture infective dose) per 25 µl is prepared using as diluent, serum-free cell culture medium containing antibiotics and fresh guinea-pig or rabbit complement at a final concentration of 10%.

iv) 25 µl of the appropriate dilution of stock virus is added to every well containing 25 µl of each serum dilution, except the test serum toxicity control wells and cell control wells on each plate.

v) A virus back titration of the working dilution of stock virus is included, using four wells per tenfold dilution, to confirm the validity of the test results.

vi) The plates are covered and shaken gently to facilitate mixing of the serum/virus mixtures.

vii) The plates are incubated for 1 hour at 37°C in a humid atmosphere of 5% CO_2 in air.

~~3 OIE Reference Laboratory for Equine viral arteritis at the Maxwell H. Gluck Equine Research Center, University of Kentucky, United States of America.~~

4 Available from the OIE Reference Laboratory for Equine viral arteritis in the United Kingdom (consult the OIE web site for full contact details: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/).

5 Available from the OIE Reference Laboratory for Equine viral arteritis in the United States of America (USA) (consult the OIE web site for full contact details: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/).

viii) A suspension of cells from 3–5-day-old cultures of RK-13 cells are prepared using a concentration that will ensure confluent monolayers in the microtitre plate wells within 18–24 hours after seeding.

ix) 100 µl of cell suspension is added to every well, the plates covered with plate lids or sealed with tape and shaken gently.

x) The plates are incubated at 37°C in a humid atmosphere of 5% CO_2 in air.

xi) The plates are read microscopically for nonviral CPE after 12–18 hours and again for viral CPE after 48–72 hours' incubation. The validity of the test is confirmed by establishing that the working dilution of stock virus contained 30–300 $TCID_{50}$ virus and that ~~the titres of~~ the positive serum controls are within 0.3 $\log_{10}$ units of their predetermined titres.

A serum dilution is considered to be positive if there is an estimated 75% or greater reduction in the amount of viral CPE in the serum test wells compared with that present in the wells of the lowest virus control dilution. End-points are then calculated using the Spearman–Kärber method. A titre of 1/4 or greater is considered to be positive. A negative serum should only have a trace (less than 25%) or no virus neutralisation at the lowest dilution tested. Antibody titres may, on occasion, be difficult to define as partial neutralisation may be observed over a range of several serum dilutions. Not infrequently, sera will be encountered that give rise to toxic changes in the lower dilutions tested. In such cases it may not be possible to establish whether the sample is negative or a low-titred positive. The problem may be overcome ~~by testing another serum sample from the animal in question or~~ by retesting the toxic sample using microtitre plates with confluent monolayers of RK-13 cells that had been seeded the previous day. Also, the toxicity in serum samples can be reduced or eliminated if the sample is adsorbed with a packed suspension of RK-13 cells prior to testing or by substituting rabbit in place of guinea-pig complement in the virus diluent. It would appear that there is more than one type of cytotoxicity in sera. Vaccination status for equine herpesviruses should be considered when evaluating sera causing non-viral cytotoxicity. One of the equine herpesvirus vaccines currently available in Europe has been shown to stimulate antibodies to rabbit kidney cells used in the vaccine production. These, in turn, can give rise to cytotoxicity, usually in the 1/4 and/or 1/8 serum but sometimes at higher dilutions, and cause difficulties in interpretation of the test results (Newton *et al*., 2004).

b) Enzyme-linked immunosorbent assay

A number of direct or indirect ELISAs have been developed for the detection of antibodies to EAV (Cho *et al*., 2000; Hedges *et al*., 1998; Kondo *et al*., 1998; Nugent *et al*., 2000). These have been based on the use of purified virus or recombinant-derived viral antigens. The usefulness of earlier assays was compromised by the frequency of false-positive reactions. The latter were associated with the presence of antibodies to various tissue culture antigens in the sera of horses that had been vaccinated with tissue-culture-derived antigens. Identification of the importance of the viral GP5 protein in stimulation of the humoral antibody response to EAV led to the development of several ELISAs that employ a portion of, or the entire recombinant protein produced in a bacterial or baculovirus expression system (Cho *et al*., 2000; Hedges *et al*., 1998). Most recently, an ovalbumin-conjugated synthetic peptide representing amino acids 81–106 of the GP5 protein has been used (Nugent *et al*., 2000). Some of these assays appear to offer nearly comparable sensitivity and specificity to the VN test and may detect EAV-specific antibodies prior to a positive reaction being obtainable in the VN test. False-negative reactions can occur, however, with some of these assays. Screening a random peptide-phage library with polyclonal sera from EAV-infected horses led to the identification of ligands, which were purified and used as antigen in an ELISA for EAV. No correlation was found, however, between absorbency values obtained with this assay and neutralising antibody titres, indicating that the antibodies being detected were largely against nonsurface epitopes of the virus. An ELISA based on the use of a combination of the GP5, M or N structural proteins of EAV expressed from recombinant baculoviruses successfully detected viral antibody in naturally or experimentally infected horses but not in EVA-vaccinated animals (Hedges *et al*., 1998). Of major importance with respect to any GP5 protein-based ELISA for EAV is the fact that test sensitivity will vary depending on the ectodomain sequence(s) of this viral protein used in the assay. Considerable amino acid sequence variation within this domain has been found between isolates of EAV. To maximise sensitivity of a GP5-based ELISA, it may be necessary to include multiple ectodomain sequences representative of known phenotypically different isolates of EAV rather than depend on a single ectodomain sequence. Two more recently described ELISAs appear to offer most promise as reliable serodiagnostic tests for EAV infection (Cho *et al*., 2000; Nugent *et al*., 2000). A blocking ELISA involving MAbs produced against the GP5 protein was reported to have a sensitivity of 99.4% and a specificity of 97.7% compared with the VN test (Cho *et al*., 2000). Another assay, a GP5 ovalbumin-conjugated synthetic peptide ELISA was shown to have a sensitivity and specificity of 96.75% and 95.6%, respectively, using a panel of 400 VN positive sera and 400 VN negative samples (Nugent *et al*., 2000). Of the number of ELISAs that have been developed (Cho *et al*., 2000; Hedges *et al*., 1998; Kondo *et al*., 1998; Nugent *et al*., 2000), few, if any, have been as extensively validated as the VN test, though some would appear to offer nearly comparable sensitivity and specificity (Cho *et al*., 2000; Hedges *et al*., 1998; Nugent *et al*., 2000). It should be noted that unlike the VN test, a positive reaction in the ELISA is not necessarily reflective of the protective immune status of an individual horse to EAV as both non-neutralising and neutralising antibodies are involved.

c) Complement fixation test

The CF test has been used in the past for diagnosing recent infection with EAV based on the fact that complement-fixing antibodies are relatively short-lived in duration (Fukunaga & McCollum, 1977). The test has been very largely superseded by the VN test and different ELISAs for carrying out serosurveillance studies and testing horses for movement.

d) Fluorescent-microsphere immunoassay

A fluorescent-MIA has been developed to detect equine antibodies to the major structural proteins of EAV (Go *et al*., 2008). It was based on cloning and expressing full-length individual major proteins, (GP5, M, N), as well as partial sequences of each structural protein and including these in separate assays. The different immunassays were analysed with a Luminex instrument. A partial GP5 protein based assay provided the best results, with sensitivity and specificity values of 92.6% and 93.9% respectively, compared with the VN test.

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

1. Background

A number of experimental and commercial vaccines have been developed against EVA. Currently, there are two commercially available vaccines, both tissue-culture derived. The first is a modified live virus (MLV) vaccine and the second an inactivated adjuvanated vaccine. The MLV vaccine is commercially available in the USA and Canada. It has also been used under ministerial control in Argentina and in New Zealand. The inactivated vaccine is licensed for commercial use in certain European countries, including Denmark, France, Germany, Hungary, Ireland, Sweden and the United Kingdom. Indications for use of these vaccines are to prevent outbreaks of EVA, including abortion in pregnant mares and establishment of the carrier state in the stallion. Since the carrier stallion is considered the principal reservoir of EAV, reduction in the carrier population would over time result in greater control over EVA and ultimately could contribute to eradication of the disease in certain countries. The MLV vaccine is prepared from virus that has been attenuated for horses by multiple serial transfers in primary equine and rabbit cells and in an equine dermal cell line (Doll *et al*., 1968; McCollum, 1970). This vaccine is licensed for use in stallions, nonpregnant mares and in nonbreeding horses. Whereas nonbreeding horses can be vaccinated at any time, stallions and mares should be vaccinated not less than 3 weeks prior to breeding. The vaccine is not recommended for use in pregnant mares, especially in the last 2 months of gestation, nor in foals under 6 weeks of age unless in the face of significant risk of exposure to natural infection. ~~The vaccine is commercially available in the USA and Canada. It has also been used in New Zealand, subject to ministerial controls, to aid in that country's EVA eradication programme.~~

The second commercially available vaccine against EVA is an inactivated product prepared from virus grown in equine cell culture, which is filtered, chemically inactivated and then combined with a metabolisable adjuvant. This vaccine is licensed for use in nonbreeding and breeding horses. In the absence of appropriate safety data, the vaccine is currently not recommended for use in pregnant mares. ~~The initial vaccination regimen involves two doses of vaccine administered intramuscularly 3–6 weeks apart. Booster vaccination at 6-month intervals is recommended by the manufacturer. The inactivated vaccine is licensed for commercial use in certain European countries, including Denmark, France, Germany, Hungary, Ireland, Sweden and the United Kingdom.~~

An additional inactivated vaccine against EVA has been developed in Japan and is kept in storage for distribution should an outbreak of EVA occur in that country. It is an aqueous formalin-inactivated vaccine that has been shown to be safe and effective for use in nonbreeding and breeding horses. For optimal immunisation with this vaccine, horses require a primary course of two injections given at an interval of 4 weeks, with a booster dose administered every 6–12 months. As the vaccine is not commercially available, no details can be provided on its production.

~~Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements.~~

2. ~~Seed management~~ Outline of production and minimum requirements for vaccines

a) Characteristics of the seed

Both MLV and inactivated commercial vaccines are derived from the prototype Bucyrus strain of EAV (ATCC VR 796), an experimentally derived variant of a foetal lung isolate recovered during an extensive outbreak of respiratory disease and abortion near Bucyrus, Ohio, USA, in 1953 (Doll *et al*., 1957). Available evidence

points to the existence of only one major serotype of the virus, and strain variation is not considered to be of significance in relation to vaccine efficacy (McCollum, 1970; Timoney & McCollum, 1993).

i) *Biological characteristics of the master seed*

In the case of the MLV vaccine, the prototype virus (ATCC VR 796) was attenuated by serial passage in primary cultures of horse kidney (HK-131), rabbit kidney (RK-111), and a diploid equine dermal cell line, ATCC CCL57 (ECID-24) (Doll *et al*., 1968; McCollum, 1970). The indications from use of this vaccine are that the virus is safe and immunogenic between its 80th and 111th passage in primary rabbit kidney cells (Doll *et al*., 1968; McCollum, 1970~~; Timoney *et al*., 1988~~).

The inactivated adjuvanted vaccine is prepared from the unattenuated prototype Bucyrus strain of EAV (ATCC VR 796) that has been plaque purified and in its fourth serial passage in the diploid equine dermal cell line (ECID-4). After growth in cell culture, the virus is then purified by filtration before being chemically inactivated and adjuvanted.

~~Suitable Lots of master seed virus for each vaccine should be maintained in liquid nitrogen or its equivalent.~~

~~b) Method of culture~~

The virus for both MLV and inactivated vaccines should be grown in a stable cell culture system, such as equine dermal cells, using an appropriate medium supplemented with sterile bovine serum or bovine serum albumin as replacement for bovine serum in the growth medium. Cell monolayers should be washed prior to virus inoculation to remove traces of bovine serum. Extensive virus growth as evidenced by the appearance of cytopathic changes in 80–100% of the cells should be obtained within 2–3 days. Lots of master seed virus for each vaccine are maintained in liquid nitrogen or its equivalent.

ii) *Quality criteria* (*sterility, purity, freedom from extraneous agents*)

Tests for sterility, purity and freedom of vaccines from contamination with extraneous agents can be found in chapter 1.1.7.

iii) *Validation as a vaccine strain*

In the case of both MLV and inactivated vaccines, the respective virus strains should be grown in an appropriate cell culture system that has been officially approved for vaccine production and confirmed to be free from extraneous bacteria, fungi, mycoplasmas and viruses (Moore, 1986). The identity of the vaccine virus in the master seed should be confirmed by neutralisation with homologous anti-EAV serum. Incomplete neutralisation of EAV by homologous horse or rabbit antisera has been scientifically documented (Moore, 1986; Senne *et al*., 1985) and is a problem when screening master seed virus for extraneous viruses and when attempting to confirm the identity of the vaccine virus. The problem has been circumvented by reducing the infectivity titre of the master seed virus below that required for seed virus production before conducting a neutralisation test on the diluted virus. Virus/serum mixtures are tested for residual live virus by serial passage in cell culture. No evidence of cytopathic viruses, haemadsorbing viruses, or noncytopathic strains of bovine virus diarrhoea virus should be found, based on attempted virus isolation in cell culture. If cells of equine origin are used, they should be confirmed to be free from equine infectious anaemia virus. ~~The newer~~ Conventional technologies such as PCR and antigen-capture ELISAs are now more commonly used than virus isolation in screening for adventitious agents.

~~The MLV vaccine has been shown to be both safe and effective for use in stallions and nonpregnant mares (Timoney *et al*., 1988). Although not recommended by the manufacturer for pregnant mares, especially in the last 2 months of gestation, the vaccine has been used to immunise pregnant mares in the face of high risk of natural exposure to EAV, with minimal, if any, reported adverse effects. Vaccination confers a high level of protective immunity that persists for at least several years (McCollum, 1970; Timoney & McCollum, 1993). Based on experimental studies and extensive field use of the vaccine since 1985, there is no evidence of back reversion to virulence of the vaccine virus, nor of recombination of the vaccine virus with naturally occurring strains of EAV. Furthermore, there is no confirmed evidence that the attenuated strain of EAV in the current vaccine localises and sets up the carrier state in the reproductive tract of the vaccinated stallion (Timoney & McCollum, 1993; 2000; Timoney *et al*., 1988).~~

~~The commercial inactivated vaccine has been shown to be nonreactive and safe for use in healthy nonbreeding and breeding horses. Transient local reactions may be observed in less than 10% of horses vaccinated with the inactivated vaccine. Limited field studies of this vaccine indicate that it is immunogenic, stimulating a satisfactory degree of immunity, the duration of which has yet to be reported.~~

~~Although there are no published reports on the efficacy of either commercial vaccine in preventing establishment of the carrier state in the stallion, an experimental aqueous formalin inactivated vaccine against EVA has been shown to prevent virus persistence in the reproductive tract of vaccinated stallions following subsequent challenge with EAV (Fukunaga *et al*., 1992).~~

b) Method of manufacture

i) *Procedure*

Both the MLV and inactivated vaccines are produced by cultivation of the respective seed viruses in an equine dermal cell system. Cell monolayers should be washed prior to inoculation with seed virus to remove traces of bovine serum in the growth medium. Inoculated cultures should be maintained on an appropriate maintenance medium. Harvesting of infected cultures should take place when almost the entire cell sheet shows the characteristic CPE. Supernatant fluid and cells are harvested and clarified of cellular debris and unwanted material by filtration. In the case of the inactivated vaccine, the purified virus is then chemically inactivated and adjuvanted with a metabolisable adjuvant. The preservatives added to the MLV and inactivated vaccines are neomycin, polymycin B and amphotericin B.

ii) *Requirements for ingredients*

Refer to chapter 1.1.6 the focus of which is on products of biological origin of negligible risk.

iii) *In-process controls*

The MLV and inactivated vaccines should be produced in a stable cell line that has been tested for identity and confirmed to be free from contamination by bacteria, fungi, mycoplasmas or other adventitious agents. In addition to the preproduction testing of the master seed virus for each vaccine and the cell line for adventitious contaminants, the cell cultures infected with the respective vaccine viruses should be examined macroscopically for evidence of microbial growth or other extraneous contamination during the incubation period. If growth in a culture vessel cannot be reliably determined by visual examination, subculture, microscopic examination, or both should be carried out.

iv) *Final product batch tests*

Sterility

Tests for sterility and freedom from contamination of biological materials can be found in chapter 1.1.7. In the case of both MLV and inactivated vaccines, each production lot of vaccine should be checked for extraneous bacterial, fungal and mycoplasmal contaminants.

Safety

The vaccine should be safety tested by the intramuscular inoculation of at least two horses seronegative for neutralising antibodies to EAV with one vaccine dose of lyophilised virus each (Moore, 1986). None of the inoculated horses should develop any clinical signs of disease other than mild pyrexia during the ensuing 2-week observation period. Transient local reactions may be observed in less than 10% of horses inoculated with either vaccine. In addition, nasopharyngeal swabs should be collected daily from each horse for attempted virus isolation; white blood cell counts and body temperatures should also be determined on a daily basis. No significant febrile or haematological changes should supervene following vaccination (Timoney & McCollum, 1993~~; Timoney *et al*., 1988~~). Limited shedding of vaccine virus by the respiratory route and in semen ~~for at most 7 days~~ may be demonstrated in the occasional horse within the first 7 days after vaccination ~~(Timoney *et al*., 1988)~~. There is no evidence of persistence of the vaccine virus in the reproductive tract of vaccinated stallions (Timoney & McCollum, 1993~~; 2000; Timoney *et al*., 1988~~).

To ensure complete inactivation of the vaccine virus, each serial lot of the inactivated vaccine should be checked for viable virus by three serial passages in equine dermal cells and by direct fluorescent antibody staining with specific EAV conjugate before being combined with adjuvant. This should be followed by a safety test in guinea-pigs and mice.

Batch potency

Potency of the vaccine in the final containers is determined by plaque infectivity assay in monolayer cultures of equine dermal cells and by a vaccination challenge test in horses (Moore, 1986). The vaccine must be tested in triplicate in cell culture, the mean infectivity titre calculated and the dose rate determined on the basis that each dose of vaccine shall contain not less than 3×10^4 plaque-forming units of attenuated EAV. The *in-vivo* potency of the MLV and inactivated vaccines is evaluated in a single vaccination challenge test using 17–20 vaccinated and 5–7 control horses or in two tests each comprising ten vaccinates and five controls. The viral antigen concentration in the inactivated vaccine is over one-thousand times the concentration of viral antigen present in the MLV vaccine.

c) Requirements for authorisation/registration/licensing

i) *Manufacturing process*

For vaccine registration, all relevant details concerning manufacture of the vaccine and quality control section should be submitted to the authorities. This information shall be provided from three consecutive batches with a volume of not less than 1/3 of the typical industrial batch volume.

ii) *Safety requirements*

The manufacturer of the MLV recommends a single dose of vaccine administered intramuscularly ~~intravenously~~ for primary vaccination followed by annual revaccination. The recommended vaccination regimen for the inactivated vaccine, which should also be administered by the intramuscular route, is a primary course of two vaccinations 3–6 weeks apart, followed by revaccination every 6 months.

- *Target and non-target animal safety*

  The MLV vaccine is considered safe for stallions and nonpregnant mares. There is no evidence to indicate that the vaccine virus can establish the carrier state in the vaccinated stallion. The MLV vaccine is not recommended for use in pregnant mares or in foals less than 6 weeks of age. Although contra-indicated by the manufacturer, this vaccine has been used in pregnant mares in the first two trimesters without any adverse sequelae. There is the risk of abortion in mares vaccinated within the last two months of gestation. The inactivated vaccine is safe for use in non-breeding and breeding animals. In the absence of appropriate safety data, the vaccine is not currently recommended for use in pregnant mares.

- *Reversion-to-virulence for attenuated live vaccines and environmental considerations*

  Both experimental and extensive field studies conducted since the MLV vaccine was first released commercially in 1985, have failed to provide any evidence of back reversion to virulence or of recombination with naturally occurring strains of EAV (Timoney & McCollum, 1993).

- *Precautions*

  The manufacturer of both the MLV and inactivated vaccines provides adequate information in the respective vaccine inserts as to the recommended usage of each vaccine, including certain contra-indications in the case of the MLV vaccine. Neither vaccine is harmful to vaccinators.

iii) *Efficacy requirements*

Both MLV and inactivated vaccines have been evaluated for efficacy in vaccination – challenge studies. This involved respiratory challenge of a group of first-time vaccinated horses 4 weeks after primary immunisation, with the virulent prototype Bucyrus strain of EAV. The level of protective immunity engendered by vaccination was assessed based on failure to produce clinical signs of EVA in the challenged horses or a significant reduction in the severity of disease compared to that observed in the nonvaccinated controls. The efficacy of vaccination in preventing establishment of the carrier state in vaccinated stallions was similarly evaluated.

iv) *Duration of immunity*

Detectable neutralising antibody titres to EAV should develop in the majority of horses within 1–2 weeks of vaccination with the MLV vaccine (Timoney & McCollum, 1993~~; Timoney *et al*., 1988~~). Reported responses to primary vaccination have been variable in a couple of studies. In one stallion vaccination study, there was a rapid fall in antibody titres with a significant number of animals reverting to seronegativity 1–3 months after vaccination ~~(Timoney *et al*., 1988)~~. On the other hand, other studies have been characterised by an excellent durable response, with persistence of high VN levels for at least 1–2 years. Revaccination with this vaccine results in an excellent anamnestic response, with the development of high antibody titres that remain relatively undiminished for several years (Timoney & McCollum, 1993).

Experimental studies have shown that most horses vaccinated with the inactivated vaccine develop low to moderate neutralising antibody titres to EAV by day 14 after the second vaccination. There is no published information on the duration of immunity conferred by this vaccine.

v) *Stability*

The lyophilised MLV vaccine can be stored for at least 3–4 years at 2–7°C without loss in infectivity, provided it is kept in the dark. Infectivity is preserved for much longer periods if vaccine is frozen at –20°C or below. Once rehydrated, however, the vaccine should be used within 1 hour or else destroyed. The inactivated vaccine is stored as a liquid suspension at 2–8°C, with no loss of potency for at least 1 year, provided it is protected from light.

~~f) Preservatives~~

~~The preservatives added to the MLV and inactivated vaccines are neomycin, polymyxin B and amphotericin B.~~

~~g) Precautions (hazards)~~

~~Pregnant mares should not be vaccinated with the MLV vaccine during the last 2 months of gestation, as there is a risk, albeit minimal, of fetal invasion by the vaccine virus. The possibility of a vaccinally induced anaphylactic reaction, though very rare, could result from the administration of either the MLV or inactivated vaccine. In the absence of appropriate safety data, the inactivated vaccine is currently not recommended for use in pregnant mares.~~

~~5. Tests on the final product~~

~~a) Safety~~

~~With the exception of the inactivated vaccine, which needs to be sterility tested a second time to ensure freedom from contamination, no further safety tests are required on the inactivated or MLV vaccines.~~

~~b) Potency~~

~~No potency tests additional to those conducted on each production lot of the MLV or inactivated vaccines are required on either final product.~~

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NB: There are OIE Reference Laboratories for Equine viral arteritis
(see Table in Part 4 of this *Terrestrial Manual* or consult the OIE web site for the most up-to-date list:
http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/ http://www.oie.int/).
Please contact the OIE Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for equine viral arteritis

CHAPTER 2.5.11.

GLANDERS

SUMMARY

Glanders is a contagious and fatal disease of horses, donkeys, and mules, ~~and is~~ caused by infection with the bacterium Burkholderia mallei (*~~the name recently changed from~~ previously named* Pseudomonas mallei *~~and was previously classified as~~* ~~Pfeifferella, Loefflerella, Malleomyces or Actinobacillus~~). *The ~~disease~~ pathogen causes nodules and ulcerations in the upper respiratory tract and lungs. A skin form also occurs, known as 'farcy'. Control of glanders requires testing of suspect clinical cases, screening of apparently normal equids, and elimination of positive reactors. ~~It~~ As* B. mallei *can be transmitted to humans, all infected/contaminated or potentially infected/contaminated material must be handled in a laboratory with appropriate biosafety and biosecurity controls following a biorisk analysis.~~*that meets the requirements for Containment Group 3 pathogens*.

Identification of the agent: *Smears from fresh material may reveal Gram-negative nonsporulating, nonencapsulated rods. The presence of a capsule-like cover has been demonstrated by electron microscopy. The bacteria grow aerobically and prefer media that contain glycerol. Unlike the* Pseudomonas *species and the closely related bacterium* B. pseudomallei, B. mallei *is nonmotile. Guinea-pigs are highly susceptible, and males can be used, if strictly necessary, to recover the organism from a heavily contaminated sample. Commercially available biochemical identification kits lack diagnostic sensitivity. Specific monoclonal antibodies and polymerase chain reaction (PCR) as well as real-time PCR assays are available. ~~The latter have also been evaluated in recent outbreaks.~~*

~~Mallein and~~ Serological tests: *Complement fixation test is an ~~and enzyme-linked immunosorbent assays are~~ ~~the most~~ accurate and reliable serological method for diagnostic use. Enzyme-linked immunosorbent assays show promise once their validation is complete. ~~The mallein test is a sensitive and specific clinical test for hypersensitivity against~~* B. mallei*~~. Mallein, a water soluble protein fraction of the organism, is injected intradermo-palpebrally or subcutaneously given as eye drop. In infected animals, the skin or the eyelid swells markedly within 1–2 days. The test is especially useful in remote endemic areas where sample transport or proper cooling of samples is not possible.~~ A Rose Bengal plate agglutination test has ~~recently~~ been developed ~~in Russia it has been validated in Russia only~~. The immunoblot test based on a crude formalin preparation of* B. mallei *antigens from isolates of different geographical regions is also a sensitive and specific assay.*

Mallein test: *The mallein test is a hypersensitivity test against* B. mallei*. The test is not generally recommended because of animal welfare concerns, however it can be useful in remote endemic areas where sample transport or proper cooling of samples is not possible. Mallein, a water soluble protein fraction of the organism, is injected intradermo-palpebrally. In infected animals, the eyelid swells markedly within 1–2 days.*

Requirements for vaccines and diagnostic biologicals: *There are no vaccines. Mallein purified protein derivative is currently available commercially. ~~from, for example, the Central Veterinary Control and Research Institute, 06020 Etlik, Ankara, Turkey and from the Pasteur Institute, Bucharest, Romania, Calea Giulesti 333, Cod:060269, Sector 6 aprovizionare@pasteur.ro~~*

A. INTRODUCTION

Glanders is a bacterial disease of perissodactyls or odd-toed ungulates. It has zoonotic potential and has been known since ancient times. It is caused by the bacterium *Burkholderia mallei* (~~the name recently changed from~~ previously known as *Pseudomonas mallei,* Yabuuchi *et al.*, 1992) and has been classified in the past as *Pfeifferella*, *Loefflerella, Malleomyces* or *Actinobacillus*. It is a serious contagious disease in equids and outbreaks ~~of the disease~~ may also occur in felids living in the wild or in zoological gardens. Susceptibility to glanders has been proved in camels, bears, wolves and dogs. Carnivores may become infected by eating infected meat, but cattle and pigs are resistant ~~(Minett, 1959)~~. Small ruminants may be infected if kept in close contact with glanderous horses (Wittig *et al.*, 2006). ~~It is accepted knowledge that~~ Glanders generally takes an ~~Glanders in the acute form occurs most frequently~~ acute form in donkeys and mules with high fever and respiratory signs (swollen nostrils, dyspnoea, and pneumonia) and death occurs within a few days. In horses, glanders generally takes a more chronic course and horses may survive for several years. Chronic and subclinical 'occult' cases are dangerous sources of infection due to the permanent or intermittent shedding of bacteria (Wittig *et al.*, 2006). Kahn *et al.* (2012) reviewed the disease, its epidemiology, diagnosis and control.

In horses, inflammatory ~~nod~~pustules and ulcers develop in the nasal ~~passages~~ conchae and nasal septae, which give rise to a sticky yellow discharge, accompanied by enlarged firm submaxillary lymph nodes. Stellate scarring follows upon healing of the ulcers. The formation of reddish nodular abscesses with a central grey necrotic zone in the lungs is accompanied by progressive debility, febrile episodes, coughing and dyspnoea. Diarrhoea and polyuria can also occur. In the skin form ('farcy'), the lymphatics are enlarged and 0.5–2.5 cm sized nodular abscesses ('buds') develop, which ulcerate and discharge yellow oily pus. Dry ulcers may also develop. Pyrogranulomatous nodules are ~~regularly~~ sometimes found in the liver and spleen (Wernery *et al.*, 2012). Discharges from the respiratory tract and skin are infective, and transmission between animals, which is facilitated by close contact, inhalation, ingestion of contaminated material (e.g. from infected feed and water troughs), or by inoculation (e.g. via a harness) is common. The incubation period can range from a few days to many months (~~Monlux, 1984;~~ Wittig *et al.*, 2006).

Glanders is transmissible to humans by direct contact with diseased animals or with infected/contaminated material. In the untreated acute disease, the mortality rate can reach 95% within 3 weeks (Neubauer *et al.*, 1997). However, survival is possible if the infected person is treated early and aggressively with multiple systemic antibiotic therapies ~~(Kahn & Ashford, 2001; Srinivasan *et al.*, 2001)~~. A chronic form with abscessation can occur (Neubauer *et al.*, 1997). When handling suspect or known infected animals or fomites, stringent precautions must ~~should~~ be taken to prevent self-infection or transmission of the bacterium ~~to other equids~~. Laboratory samples must ~~should~~ be securely packaged, kept cool (not frozen) and shipped as outlined in Chapter 1.1.1 *Collection and shipment of diagnostic specimens*. All manipulations with potentially infected/contaminated material must be performed ~~in a laboratory that meets the requirements for Containment Group 3 pathogens as outlined in Chapter 1.1.3 *Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities.*~~ at an appropriate biosafety and containment level determined by biorisk analysis (see Chapter 1.1.3 *Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities*).

Glanders has been eradicated from many countries by statutory testing, culling ~~elimination~~ of infected animals, and import restrictions. It persists in numerous ~~some~~ Asian, African and South American countries and can be considered a re-emerging disease. Glanders can be ~~easily~~ introduced into glanders-free areas by pet or racing equids (Neubauer *et al.*, 2005).

B. DIAGNOSTIC TECHNIQUES

Table 1. *Test methods available for glanders and their purpose*

Method	**Purpose**				
	Population freedom from infection	Individual animal freedom from infection	Efficiency of eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance
Complement fixation	±	±	+++	±	+++
Western blotting	±	±	++	±	++
ELISA	±	±	++	±	++
Malleinisation	±	±	±	±	±
PCR	=	=	=	±	=

Method	Purpose				
	Population freedom from infection	Individual animal freedom from infection	Efficiency of eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance
Animal inoculation	≡	≡	≡	±	≡
Culture	≡	≡	≡	±	≡
~~Necropsy~~	~~–~~	~~–~~	~~–~~	~~++~~	~~–~~

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose.
Although not all of the tests listed as category +++ or ++ have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.
ELISA = enzyme-linked immunosorbent assay; PCR = polymerase chain reaction.

1. Identification of the agent

Cases for specific glanders investigation should be differentiated on clinical grounds from other chronic infections affecting ~~of~~ the nasal mucous membranes ~~mucosae~~ or sinuses. ~~and from~~ Among these are strangles (*Streptococcus equi* ~~infection~~), ulcerative lymphangitis (*Corynebacterium pseudotuberculosis*), pseudotuberculosis (*Yersinia pseudotuberculosis*) and sporotrichosis (*Sporotrichium* spp.). Glanders should be unmistakably ~~positively~~ excluded from suspected cases of epizootic lymphangitis (~~caused by~~ *Histoplasma farciminosum*), with which it has many clinical similarities. In humans in particular, glanders should be distinguished from melioidosis ~~(*B. pseudomallei* infection), which~~, caused by ~~an organism with close similarities to~~ *B. pseudomallei* a bacterium closely related to *B. mallei* ~~(Minett, 1959)~~.

a) Morphology of *Burkholderia mallei*

The organisms are fairly numerous in smears from fresh lesions, but scarce in older lesions ~~(Wilson & Miles, 1964)~~. Smears should be stained with methylene blue or Gram stain. The ~~organisms are mainly extracellular, fairly straight~~ Gram-negative rods have rounded ends, are 2–5 µm long and 0.3–0.8 µm wide with granular inclusions of various size. ~~They often~~ The bacteria are generally located extracellularly and frequently stain irregularly and poorly when Gram stain is used. They do not have a readily visible capsule under the light microscope and do not form spores. The presence of a capsule-like cover has been verified ~~established~~ by electron microscopy. This capsule is composed of neutral carbohydrates and serves to protect the cell from unfavourable environmental factors. Unlike other organisms in the *Pseudomonas* group and its close relative *B. pseudomallei*, *B. mallei* has no flagellae and is therefore nonmotile (~~Krieg & Holt, 1984;~~ Sprague & Neubauer, 2004). Nonmotility is the most important phenotypic characteristic diagnostically and must be demonstrated when pure culture is available. The organisms are difficult to detect ~~demonstrate~~ in tissue sections, where they may have a beaded appearance. In culture media, they vary in appearance depending on the age of the culture and type of medium. In older cultures, there is much pleomorphism. Branching filaments form on the surface of broth cultures (Neubauer *et al.*, 2005).

b) Cultural characteristics

It is preferable to attempt isolation from unopened, uncontaminated lesions ~~(Miller *et al.*, 1948)~~. The organism is aerobic and facultative anaerobic only in the presence of nitrate ~~(Gilardi, 1985; Krieg & Holt, 1984)~~, growing optimally at 37°C ~~(Mahaderan *et al.*, 1987)~~. It grows well, but slowly, on ordinary culture media, including sheep blood agar. 72-hour incubation of cultures is recommended; glycerol enrichment is particularly useful. The tiny greyish shiny colonies of *B. mallei* on sheep blood agar can be easily overgrown by other bacteria; hence careful observation is needed not to overlook the bacteria after 72 hours of incubation. After a few days on glycerol agar, a confluent, smooth, moist and slightly viscous cream coloured growth can be observed. On continued incubation, the growth thickens and becomes dark brown and tough. *Burkholderia mallei* also grows well on glycerol potato agar and in glycerol broth, on which a slimy pellicle forms. On plain nutrient agar, the growth is much less ~~luxuriant~~ effusive, and growth is poor on gelatine ~~(Steele, 1980)~~. Various commercially available *Burkholderia* selective agars enable the growth of *B. mallei* (Glass *et al.*, 2009). Even in fresh samples obtained under sterile conditions *B. mallei* is ~~regularly~~ often overgrown by other bacteria, which makes isolation extremely difficult (Wernery, 2009).

Growth characteristics may alter *in vitro*, so fresh isolates should be used for identification reactions. ~~Litmus milk is slightly acidified by *B. mallei*, and coagulation may occur after long incubation. The organism reduces nitrates. Although some workers researchers have claimed that glucose is the only carbohydrate that is fermented (slowly and inconstantly), others have shown that if an appropriate medium and indicator are~~

used, glucose and other carbohydrates, such as arabinose, fructose, galactose and mannose, are consistently fermented by *B. mallei* (Evans, 1966). The positive biochemical reactions include reduction of nitrates, utilisation of arginine by arginine dihydrolase, assimilation of glucose, N-acetyl glucosamine and gluconate. Strain to strain variation is observed in the assimilation reactions of arabinose, fructose, mannose, mannitol, adipic acid, malate, trisodium citrate, phenyl acetic acid and VP reaction, which needs an incubation time of 48 hours. Indole is not produced, horse blood is not haemolysed and no diffusible pigments are produced in cultures (Krieg & Holt, 1984). Commercially available laboratory test biochemical identification systems (e.g. API [Analytical Profile Index] system: Analytab Products, BioMerieux or Biolog [Hayward, California]) can be used for easy confirmation that an organism belongs to the *Pseudomonas* group. In general, however, commercially available systems are not suitable for unambiguous identification of members of the steadily growing number of species within the genus *Burkholderia* (Glass & Popovic, 2005). Lack of motility is therefore of special relevance. A bacteriophage specific for *B. mallei* is available (Woods *et al.*, 2002).

All prepared culture media should be subjected to quality control and must support growth of the suspect organism from a small inoculum. The reference strain should be cultured in parallel with the suspicious samples to ensure that the tests are functioning correctly.

In contaminated samples, supplementation of media with substances that inhibit the growth of Gram-positive organisms (e.g. crystal violet, proflavine) has proven to be useful, as well as pre-treatment with penicillin (1000 units/ml for 3 hours at 37°C) (Minett, 1959). A semi-selective medium (Xie *et al.*, 1980) composed of polymyxin E (1000 units), bacitracin (250 units), and actidione (0.25 mg) incorporated into nutrient agar (100 ml) containing glycerine (4%), donkey or horse serum (10%), and ovine haemoglobin or tryptone agar (0.1%) has been developed. Heavily contaminated samples should also be streaked onto stiff blood agar (3% agar) which inhibits the growth of *Proteus* spp., and onto Sabouraud dextrose agar which inhibits the growth of many Gram-positive and Gram-negative bacteria in glanders samples. These samples should also be streaked onto normal blood agar and incubated for 24 hours anaerobically to inhibit the growth of obligate aerobes. Isolation of *B. mallei* from the anaerobic plates needs a further 24 hours' incubation at 37°C. PCR methods may also prove useful for testing contaminated samples.

Outside the body, the organism shows little resistance to drying, heat, light or chemicals, so that survival beyond 2 weeks is unlikely (Neubauer *et al.*, 1997). Under favourable conditions, however, it can probably survive a few months. *Burkholderia mallei* can remain viable in tap water for at least 1 month (Steele, 1980). For disinfection, benzalkonium chloride or 'roccal' (1/2,000), sodium hypochlorite (500 ppm available chlorine), iodine, mercuric chloride in alcohol, and potassium permanganate have been shown to be highly effective against *B. mallei* (Mahaderan *et al.*, 1987). Phenolic disinfectants are less effective St Georgiev, (2008). The guidelines for handling and application of disinfectants in the respective countries must be observed.

c) Laboratory animal inoculation

Guinea-pigs, hamsters and cats have been used for diagnosis when necessary. If isolation in a laboratory animal is considered unavoidable, suspected material is inoculated intraperitoneally into a male guinea-pig. As this technique has a sensitivity of only 20%, the inoculation of at least five animals is recommended (Neubauer *et al.*, 1997). Positive material will cause a severe localised peritonitis and orchitis (the Strauss reaction). The number of organisms and their virulence determines the severity of lesions. Steps are used if the test material is heavily contaminated (Gould, 1950). The Strauss reaction is not specific for glanders and other organisms can elicit it. Bacteriological examination of infected testes should confirm the specificity of the response obtained.

c) Confirmation Identification of *Burkholderia mallei* by polymerase chain reaction and real-time PCR

In the past few years, several PCR and real-time PCR assays for the identification of *B. mallei* have been developed (Bauernfeind *et al.*, 1998; Lee *et al.*, 2005; Sprague *et al.*, 2002; Thibault *et al.*, 2004; Ulrich *et al.*, 2006; U'Ren *et al.*, 2005), but only one conventional PCR and one real-time PCR assay were evaluated using samples from a recent outbreak of glanders in horses (Scholz *et al.*, 2006; Tomaso *et al.*, 2006). These two assays will therefore be described in more detail, but inter-laboratory studies are needed to confirm the robustness of these assays. The guidelines and precautions outlined in Chapter 1.1.5 *Principles and methods of validation of diagnostic assays for infectious diseases* should be observed have to be taken into account.

- **DNA preparation**

Single colonies are transferred from an agar plate to 200 µl deionised water. After heat inactivation (for example 99°C for 30 minutes), the DNA isolation can be performed using commercial DNA preparation kits for gram negative bacteria (see Scholz *et al.*, 2006 and Tomas *et al.*,2006). lysis buffer (5× buffer D [PCR

~~Optimation Kit, Invitrogen, DeShelp, The Netherlands, 1/5 diluted in ultra-pure water]; 0.5% Tween 20 [ICI, American Limited, Merck, Hohenbrunn, Germany]; 2 mg/ml proteinase K [Roche Diagnostics, Mannheim, Germany]). After incubation at 56°C for 1 hour and inactivation for 10 minutes at 95°C 2 and 4 µl of the cleared lysate are used as template in the PCR or the real-time PCR assay, respectively.~~Alternatively, heat-inactivated bacteria from pure cultures (99°C, 10 minutes) can be used directly for PCR reaction.

Tissue samples from horses (skin, ~~liver, spleen,~~ lung, mucous membrane of the nasal conchae and septae, liver and spleen) that have been inactivated and preserved in formalin (48 hours, 10% v/v) are cut with a scalpel into pieces of 0.5 × 0.5 cm (approximately 500 mg). The specimens are washed twice in deionised water (10 ml), incubated overnight in sterile saline at 4°C, and minced by freezing in liquid nitrogen, followed by grinding with a mortar and pestle. Total DNA is prepared from 50 mg tissue using a commercial extraction kit ~~the QIAamp Tissue Kit~~™ according to the manufacturer's instructions ~~(Qiagen, Hilden, Germany)~~. DNA is eluted with 80 µl dH_2O or as appropriate for the kit used ~~of which~~ 4 µl eluate is used as template.

- **PCR assay (Scholz *et al.*, 2006)**

The assay may have to be adapted to the PCR instrument used with minor modifications to the cycle conditions and the concentration of the ~~chemicals~~ reagents used.

The oligonucleotides used by Scholz *et al.,* (2006) are based on the differences between the *fliP* sequences from *B. mallei* ATCC 23344^T (accession numbers NC_006350, NC_006351) and *B. pseudomallei* K96243 (accession numbers NC_006348, NC_006349). Primers Bma-IS407-flip-f (5'-TCA-GGT-TTG-TAT-GTC-GCT-CGG-3') and Bma- IS407-flip-r (5'-CTA-GGT-GAA-GCT-CTG-CGC-GAG-3') are used to amplify a 989 bp fragment. The PCR uses 50 µl ready-to-go master mix ~~(Eppendorf, Hamburg, Germany)~~ and 15 pmol of each primer. Thermal cycling conditions are 94°C for 30 seconds and 35 cycles at 65°C for 30 seconds and 72°C for 60 seconds and succeeded by a final elongation step at 72°C for 7 minutes. Visualisation of the products takes place under UV light after agarose gel (1% w/v in TAE buffer) electrophoresis and staining with ethidium bromide. No template controls containing PCR-grade water instead of template and positive controls containing *B. mallei* DNA have to be included in each run to detect contamination by amplicons of former runs or amplification failure.

The lower detection limit of this assay is 10 fg or 2 genome equivalents.

- **Real-time PCR assay (Tomaso *et al.*, 2006)**

The assay should be adapted to the real-time PCR instrument used ~~with minor modifications~~, e.g. the cycling vials should be chosen according to the manufacturer's recommendations, the concentration of the oligonucleotides may have to be ~~doubled~~ increased, or the labelling of the probes altered. ~~The authors used a MX3000P™ real-time PCR system (Stratagene, Amsterdam, Netherlands) and 96-well plates (ThermoFast 96 ABGene™, Rapidozym, Berlin, Germany).~~

The oligonucleotides used in Tomaso *et al.* (2006) are based on differences in the *fliP* sequences of *B. mallei* ATCC 23344^T (accession numbers NC_006350, NC_006351) and *B. pseudomallei* K96243 (accession numbers NC_006348, NC_006349). The fluorogenic probe is synthesised with 6-carboxy-fluorescein (FAM) at the 5'-end and black hole quencher 1 (BHQ1) at the 3'-end. Oligonucleotides used were Bma-flip-f (5'-CCC-ATT-GGC-CCT-ATC-GAA-G-3'), Bma-flip-r (5'-GCC-CGA-CGA-GCA-CCT-GAT-T-3') and probe Bma-flip (5'-6FAM-CAG-GTC-AAC-GAG-CTT-CAC-GCG-GAT-C-BHQ1-3').

The 25 µl reaction mixture consists of 12.5 µl 2× ~~TaqMan™ Universal~~ master mix ~~(Applied Biosystems, Foster City, USA)~~, 0.1 µl of each primer (10 pmol/µl), 0.1 µl of the probe (10 pmol/µl) and 4 µl sample. Thermal cycling conditions are 50°C for 2 minutes; 95°C for 10 minutes; 45 ~~(50)~~ cycles at 95°C for 25 seconds and 63°C for 1 minute. Possible contaminations with amplification products from former reactions are inactivated by an initial incubation step using uracil *N*'-glycosilase.

The authors suggest including an internal inhibition control based on a bacteriophage lambda gene target (Lambda-F [5'-ATG-CCA-CGT-AAG-CGA-AAC-A-3] Lambda-R [5'-GCA-TAA-ACG-AAG-CAG-TCG-AGT-3'], Lam-YAK [5'-YAK-ACC-TTA-CCG-AAA-TCG-GTA-CGG-ATA-CCG-C-DB-3']), which can be titrated to provide reproducible cycle threshold values. However, depending on the sample material a house keeping gene targeting PCR may be used additionally or as an alternative. No template controls containing 4 µl of PCR-grade water instead and positive controls containing DNA of *B. mallei* have to be included in each run to detect amplicon contamination or amplification failure.

The linear range of the assay was determined to cover concentrations from 240 pg to 70 fg bacterial DNA/reaction. The lower limit of detection defined as the lowest amount of DNA that was consistently detectable in three runs with eight measurements each is 60 fg DNA or four genome equivalents (95% probability). The intra-assay variability of the *fliP* PCR assay for 35 pg DNA/reaction is 0.68% (based on Ct

values) and for 875 fg 1.34%, respectively. The inter-assay variability for 35 pg DNA/reaction is 0.89% (based on Ct values) and for 875 fg DNA 2.76 %, respectively.

To date, a positive result confirms the diagnosis '*Burkholderia mallei*' for an isolate and the diagnosis 'glanders' in clinical cases. It has to be kept in mind, however, that future genetic evolution may well result in *B. mallei* clones that can no longer be detected by these standard PCRs. The sensitivity of the PCR assays for clinical samples is unknown. A negative result therefore, is no proof of the absence of *B. mallei* in the sample and other diagnostic means must be applied for confirmation.

d) Laboratory animal inoculation

Animal inoculation is not recommended, because of welfare concerns. If isolation in a laboratory animal is considered unavoidable, suspected material is inoculated intraperitoneally into male guinea-pigs. As this technique has a sensitivity of only 20%, the inoculation of at least five animals is recommended (Neubauer *et al.*, 1997). Positive material will cause a severe localised peritonitis and orchitis (the Strauss reaction). The number of organisms and their virulence determines the severity of lesions. Additional pre-treatment steps have to be used if the test material is heavily contaminated. The Strauss reaction is not specific for glanders and can be provoked by other organisms, therefore *B. mallei* must be cultured from the infected testes.~~Bacteriological examination of infected testes must confirm the specificity of the response obtained.~~

e) Other methods

~~The genome of the *B. mallei* type strain ATCC 23344^{T} was sequenced in 2004 (Nierman *et al.*, 2004). Several genomes of other isolates followed and revealed a wide genomic plasticity. Passages in different host species or culture media can provoke considerable sequence alterations (Romero *et al.*, 2006). The loss of the ability to produce lipopolysaccharide (LPS) and/or capsule polysaccharide due to the occurrence of mutations during cultivation is a well-known fact and can lead to reduced or loss of virulence and thus influences serologic tests (Neubauer *et al.*, 2005). Several~~ Molecular typing techniques ~~have been successfully introduced. Simple molecular techniques like~~ such as PCR-restriction fragment length polymorphism (Tanpiboonsak *et al.*, 2004), pulsed field gel electrophoresis (Chantratita *et al.*, 2006), ~~can be used for the further discrimination of isolates.~~ ribotyping ~~using restriction enzymes *Pst*I and *Eco*RI in combination with an *E. coli* 18-mer rDNA probe produced 17 distinct ribotypes from 25 *B. mallei* isolates~~ (Harvey & Minter, 2005) or multilocus sequence typing (MLST) (Godoy *et al.*, 2003) ~~These techniques~~ are ~~still in-house tests of~~ only appropriate for use in specialised laboratories ~~as extensive strain collections are necessary. multilocus sequence typing (MLST) can be done with purified DNA thereby avoiding excessive cultivation of the agent or the maintenance of strain collections. Web-based analyses might even enhance diagnostics~~ typing ~~(Godoy *et al.*, 2003). No specific histopathological features can be described for lesions caused by *B. mallei.* For immunuhistochemical analysis, *B. mallei* specific hyperimmune sera of rabbits can be used.~~

2. Serological tests ~~and the mallein test~~

a) Complement fixation test in horses, donkeys, and mules (a prescribed test for international trade)

~~Although not as sensitive as the mallein test,~~ The CFT is an accurate serological test that has been used for many years for diagnosing glanders ~~(Blood & Radostits, 1989). It is reported to be 90–95% accurate serum being.~~ It will deliver positive results within 1 week post-infection and ~~remaining positive in the case of exacerbation of the~~ will also recognise sera from exacerbated chronic ~~process (Steele, 1980)~~ cases. Application of rigorous quality control in the formulation of CFT antigens, complement and haemolytic systems are crucial for the performance of this test as its specificity and sensitivity are critically dependent on the antigen used (Elschner *et al.*, 2011; Khan *et al.*, 2011). Recently, however, the specificity of CF testing has been questioned (Neubauer *et al.*, 2005). The CFT is ~~only~~ valid for horses, ~~(and~~ mules and camels; if used in donkeys particular care is needed to avoid misdiagnosis.

- **Antigen preparation ~~(Kelser & Reynolds, 1935)~~**

~~i) Flasks of beef infusion broth with 3% glycerol are inoculated with log-phase growth *B. mallei* and incubated at 37°C for 8–12 weeks.~~

i) The stock culture strain of *B.mallei* (Dubai 7) stored at –80°C is revived by plating onto sheep blood agar and incubated at 37°C for 48 hours to get a confluent growth.

ii) From this 48 hours culture, a loopful (0.5 mm diameter) is inoculated to 5 ml of brain–heart infusion (BHI) broth with 3% glycerol and incubated at 37°C for 24 hours.

iii) 1 ml from the above culture broth is further inoculated to 100 ml BHI broth with 3% glycerol and incubated at 37°C for 48 hours with gentle agitation.

iv) The cultures are inactivated by exposing the flasks to flowing steam (100°C) for 60 minutes.

viii) The clear supernatant is decanted and filtered. The filtrate is heated again by exposure to live steam for 1 hour ~~75 minutes on 3 consecutive days~~, and clarified by centrifugation at 3000 rpm for 10 minutes.

ivi) The clarified product is ~~concentrated to 1/10 of the original volume by evaporation on a steam or hot water bath.~~

~~v)~~ stored as concentrated antigen ~~is bottled~~ in brown glass bottles to shield from light and stored at 4°C. Antigen has been shown to be stable for at least 10 years in this concentrated state.

vii) ~~Lots~~ Aliquots of antigen are prepared by diluting the concentrated antigen 1/20 with sterile physiological saline containing ~~with~~ 0.5% phenol. The diluted antigen is dispensed into brown-glass vials and stored at 4°C. The final working dilution is determined by a block titration. The final working dilution for the CFT is prepared when performing the test.

The resulting antigen consists primarily of lipopolysaccharides (LPS). An alternative procedure is to use young cultures by growing the organism on glycerol–agar slopes for ~~12~~ up to 48 hours and washing them off with normal saline. A suspension of the culture is heated for 1 hour at 70°C and the heat-treated bacterial suspension is used as antigen. The disadvantage of this antigen preparation method is that the antigen contains all the bacterial cell components. The antigen should be safety tested by inoculating blood agar plates.

- **CFT ~~test~~ procedure ~~(NVSL, 2006)~~**

i) Serum is diluted 1/5 in veronal (barbiturate) buffered saline containing 0.1% gelatine (VBSG) or CFD (complement fixation diluent – available as tablets) without gelatine or other commercially provided CFT buffers.

ii) Diluted serum is inactivated for 30 minutes at ~~56~~ 58 ~~–60°C±2~~°C. ~~The USDA complement fixation protocol calls for inactivation for 35 minutes [National Veterinary Services Laboratories, 2006].~~ Serum of equidae other than horses should be inactivated at 63°C for 30 minutes. Camel serum is inactivated for 30 minutes at 56°C.

iii) Twofold dilutions of the sera are prepared using veronal buffer or alternative commercially available CFT buffers. in 96-well round-bottom microtitre plates.

iv) Guinea-pig complement is diluted in the chosen buffer and 5 (or optionally 4) complement haemolytic units-50% (CH_{50}) are used.

v) Sera, complement and antigen are mixed in the plates and incubated for 1 hour at 37°C. ~~An alternate acceptable~~ An alternative procedure is overnight incubation at 4°C.

vi) A 3% suspension of sensitised washed sheep red blood cells is added. ~~(The USDA protocol calls for confirmation of positive reactions in a tube test using 3% sheep red blood cells [National Veterinary Services Laboratories, 2006].)~~

vii) Plates are incubated for 45 minutes at 37°C, and then centrifuged for 5 minutes at 600 ***g***.

When using commercially available CFT-antigens and ready-to-use CFT reagents, the manufacturers' instructions should be applied.

Recommended controls to verify test conditions:

Positive control: a control serum that gives a positive reaction;

Negative control serum: a control serum that gives a negative reaction;

Anti-complementary control (*serum control*): diluent + inactivated test serum + haemolytic system;

Antigen control: diluent + antigen + complement + haemolytic system;

Haemolytic system control: diluent + haemolytic system;

Complement control: diluent + complement titration + antigen + haemolytic system.

- **Reading the results**

The absence of anti-complementary activity must be checked for each serum; anti-complementary sera must be excluded from analyses. A sample that produces 100% haemolysis at the 1/5 dilution is negative, 25–75% haemolysis is suspicious, and no haemolysis (100% fixation) is positive. Unfortunately, false-positive results can occur, and animals can remain positive for months. Moreover, *B. pseudomallei* and *B. mallei* cross react and cannot be differentiated by serology (~~Blood & Radostits, 1989;~~ Neubauer *et al.*, 1997).

It must also be kept in mind that ~~Also~~ healthy ~~horses~~ non-glanderous equids can show a false positive CF reaction for a variable period of time following a mallein intradermal test.

b) Enzyme-linked immunosorbent assays

Both plate and membrane ~~(blot)~~ based ELISAs have been used for the serodiagnosis of glanders, but none of these procedures has been able to differentiate ~~serologically~~ between *B. mallei* and *B. pseudomallei*. ~~Blotting approaches have involved both dipstick dot-blot and electrophoretically separated and transferred western blot methods (Katz *et al.*, 1999; Verma *et al.*, 1990).~~ An avidin–biotin dot ELISA has been described, but has not yet been widely used or validated. The antigen used is a concentrated and purified heat-inactivated bacterial culture. A spot of this antigen is placed on a nitrocellulose dipstick. Using antigen-dotted, pre-blocked dipsticks, the test can be completed in approximately 1 hour. An I-ELISA was shown to be of limited value for the serological diagnosis of glanders (Sprague *et al.*, 2009). An I-ELISA based on recombinant *Burkholderia* intracellular motility A protein (rBimA) showed a promising sensitivity of 100% and a specificity of 98.88% (Kumar *et al.*, 2011). A C-ELISA that makes use of an uncharacterised anti-LPS MAb has also been developed and found to be similar to the CF test in performance (Katz *et al.*, 2000). The C-ELISA was used again on a panel of horse sera originating mainly from Middle Eastern countries (Sprague *et al.*, 2009). A commercially available C-ELISA has recently been developed using anti-s *B. mallei* LPS MAb along with antigen prepared from a regional *B. mallei* isolate. This showed higher sensitivity than CFT in identifying field cases. The C-ELISA has been evaluated on donkey sera and reliable results obtained in an infection trial (Altemann, in preparation). ~~The number of positive sera investigated, however, was very limited.~~ Continuing development of monoclonal antibody reagents specific for *B. mallei* antigenic components will offer the possibility to develop more specific ELISAs ~~in the foreseeable future~~ that will help to resolve questionable test results of quarantined imported horses (~~Burtnick *et al.*, 2002; Feng *et al.*, 2006; Khrapova *et al.*, 1995;~~ Neubauer *et al.*, 1997).

None of these tests has been fully validated to date. However, a C-ELISA based on one or more *B. mallei*-specific antibodies and an I-ELISA making use of (recombinant) *B. mallei*-specific antigens have the potential to be used as alternative tests after their validations have been completed.

~~**c) The mallein test**~~

~~The mallein purified protein derivative (PPD), which is available commercially, is a solution of water-soluble protein fractions of heat-treated *B. mallei*. The test depends on infected horses being hypersensitive to mallein. Advanced clinical cases in horses and acute cases in donkeys and mules may give inconclusive results requiring additional methods to be employed (Allen, 1929).~~

~~• **The intradermo-palpebral test**~~

~~This is the most sensitive, reliable and specific test for detecting infected perissodactyls or odd-toed ungulates, and has largely displaced the ophthalmic and subcutaneous tests (Blood & Radostits, 1989): 0.1 ml of concentrated mallein PPD is injected intradermally into the lower eyelid and the test is read at 24 and 48 hours. A positive reaction is characterised by marked oedematous swelling of the eyelid, and there may be a purulent discharge from the inner canthus or conjunctiva. This is usually accompanied by a rise in temperature. With a negative response, there is usually no reaction or only a little swelling of the lower lid.~~

c) Immunoblot assays

An immunoblot assay was developed for the serodiagnosis of glanders, but further validation was impossible because of the lack of a positive serum control panel (Katz *et al.*, 1999). Recently, the development of an immunoblot using *B. mallei* LPS antigen was reinitiated. The aim was to obtain a more sensitive test than the CFT in order to retest false positive CFT sera in non-endemic areas (Elschner *et al.*, 2011). The developed assay is based on crude antigen preparations of the *B. mallei* strains Bogor, Zagreb and Mukteswar, which are also the basis of most CFT antigen formulations. The antigens are separated by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and subsequently transferred to nitrocellulose membranes. Anti-*B. mallei* LPS antibodies in a serum sample reacting to the antigen on the blot strip are visualised by animal species-specific (phosphatase) conjugate and the NBT-BCIP (Nitro blue tetrazolium-5-bromo-4-chloro-3-indolyl-phosphate) colour system. The immunoblot is scored positive if the banding pattern of the *B. mallei* LPS ladder within the 20–60 kDa region is clearly visible, suspicious if a weak colour reaction is detected and negative if no reaction is seen. 171 sera of glanderous horses and mules from Pakistan and Brazil and 305 sera of negative German horses were investigated and a sensitivity and specificity, both of 100% were found. For the time being, this test is the best evaluated serological test available. ~~As such it will be the most promising candidate to become officially recognised in the near future.~~ It has to be stressed that this test is not able to differentiate glanders from melioidosis infection and that it has not yet been evaluated for use in donkeys because of the lack of a significant number of positive control sera.

d) Other serological tests

~~The avidin–biotin dot ELISA has been described (Verma *et al.*, 1990), but has not yet been widely used or validated. The antigen is heat-inactivated bacterial culture that has been concentrated and purified. A dot of this antigen is placed on a nitrocellulose dipstick that is then used to test for antibody against *B. mallei* in equine serum. Using antigen-dotted, preblocked dipsticks, the test can be completed in approximately 1 hour. Serum or whole blood can be used for the test, and partial haemolysis does not impart any background colour to the antigen-coated area on the nitrocellulose. Recently, polysaccharide microarray technology has offered a new promising approach to improve sensitivity in serology (Parthasarathy *et al.*, 2006).~~

The Rose Bengal plate agglutination test (RBT) has been described for the diagnosis of glanders in horses and other susceptible animals; and has been validated ~~only~~ in Russia. In a study in Pakistan the RBT showed a sensitivity of 90% and a specificity of 100% (Naureen *et al.*, 2007). The antigen is a heat-inactivated bacterial suspension coloured with Rose Bengal, which is used in a plate agglutination test.

The accuracy of other agglutination and precipitin tests is unsatisfactory for control programmes. Horses with chronic glanders and those in a debilitated condition give negative or inconclusive results.

3. Tests for cellular immunity

a) The mallein test

The mallein purified protein derivative (PPD), which is available commercially, is a solution of water-soluble protein fractions of heat-treated *B. mallei*. See section C below for details of its preparation and availability. The test is not generally recommended because of animal welfare concerns, however it can be useful in remote endemic areas where sample transport or proper cooling of samples is not possible. It depends on infected horses being hypersensitive to mallein. Advanced clinical cases in horses and acute cases in donkeys and mules may give inconclusive results requiring additional diagnostic methods.

The intradermo-palpebral test is the most sensitive, reliable and specific mallein test for detecting infected perissodactyls or odd-toed ungulates, and has largely displaced other methods. 0.1 ml of concentrated mallein PPD is injected intradermally into the lower eyelid and the test is read at 24 and 48 hours. A positive reaction is characterised by marked oedematous swelling of the eyelid, and there may be a purulent discharge from the inner canthus or conjunctiva. This is usually accompanied by a rise in temperature. With a negative response, there is usually no reaction or only a little swelling of the lower lid.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

No vaccines are available.

Mallein PPD ~~for use in performing the intradermo-palpebral and ophthalmic tests~~ is available commercially[1]. ~~from e.g. the Central Veterinary Control and Research Institute, 06020 Etlik, Ankara, Turkey and the Pasteur Institute, Bucharest, Romania, Calea Giulesti 333, Cod:060269, Sector 6 *aprovizionare@pasteur.ro*. ID Lelystad has provided~~ The following information outlines the requirements for the production of mallein PPD.

1. Seed management

Three strains of *Burkholderia mallei* are employed in the production of mallein PPD, namely Bogor strain (originating from Indonesia), Mukteswar strain (India) and Zagreb strain (Yugoslavia). The seed material is kept as a stock of freeze-dried cultures. The strains are subcultured on to glycerol agar at 37°C for 1–2 days. For maintaining virulence and antigenicity, the strains may be passaged in guinea-pigs.

2. ~~Method of manufacture~~ Production

Dorset-Henley medium, enriched by the addition of trace elements, is used for the production of mallein PPD. The liquid medium is inoculated with a thick saline suspension of *B. mallei*, grown on glycerol agar. The production medium is incubated at 37°C for about 10 weeks. The bacteria are then killed by steaming for 3 hours in a Koch's steriliser. The fluid is then passed through a layer of cotton wool to remove coarse bacterial clumps. The resulting turbid fluid is cleared by membrane filtration, and one part trichloroacetic acid 40 % is immediately added to nine parts culture filtrate. The mixture is allowed to stand overnight during which the light brownish to greyish precipitate settles.

1 Central Veterinary Control and Research Institute, 06020 Etlik, Ankara, Turkey; Pasteur Institute, Bucharest, Romania, Calea Giulesti 333, Cod:060269, Sector 6 *aprovizionare@pasteur.ro*

The supernatant is decanted ~~pipetted off~~ and discarded. The precipitate is centrifuged for 15 minutes at 2500 ***g*** and the layer of precipitate is washed three or more times in a solution of 5% NaCl, pH 3, until the pH is 2.7. The washed precipitate is dissolved by stirring with a minimum of an alkaline solvent. The fluid is dark brown and has a pH of 6.7. This mallein concentrate is centrifuged again and the supernatant diluted with an equal amount of a glucose buffer solution. The protein content of this product is estimated by the Kjeldahl method and freeze-dried after it has been dispensed into ampoules.

3. In-process control

During the period of incubation, the flasks are inspected regularly for any signs of contamination, and suspicious flasks are discarded. A typical growth of the *B. mallei* cultures comprises turbidity, sedimentation, some surface growth with a tendency towards sinking, and the formation of a conspicuous slightly orange-coloured ring along the margin of the surface of the medium.

4. Batch control

Each batch of mallein PPD is tested for sterility, safety, preservatives, protein content and potency.

Sterility testing is performed according to the European Pharmacopoeia guidelines.

The examination for safety is conducted on five to ten normal healthy horses by applying the intradermo-palpebral test. The resulting swelling should be, at most, barely detectable and transient, without any signs of conjunctival discharge.

Preparations containing phenol as a preservative should not contain more than 0.5% (w/v) phenol. The protein content should be no less than 0.95 mg/ml and not more than 1.05 mg/ml.

Potency testing is performed in guinea-pigs and horses. The animals are sensitised by subcutaneous inoculation with a concentrated suspension of heat-killed *B. mallei* in paraffin oil ~~or incomplete Freund's~~ adjuvant. Cattle can also be used instead of horses. The production batch is bio-assayed against a standard mallein PPD by intradermal injection in 0.1 ml doses in such a way that complete randomisation is obtained.

In guinea-pigs, the different areas of erythema are measured after 24 hours, and in horses the increase in skin thickness is measured with callipers. The results are statistically evaluated, using standard statistical methods for parallel-line assays.

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* *

NB: There are OIE Reference Laboratories for Glanders
(see Table in Part 4 of this *Terrestrial Manual* or consult the OIE web site for the most up-to-date list:
http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/).
Please contact the OIE Reference Laboratories for any further information on
diagnostic tests, reagents and diagnostic biologicals for glanders

CHAPTER 2.5.13.

VENEZUELAN EQUINE ENCEPHALOMYELITIS

SUMMARY

Venezuelan equine encephalomyelitis (*VEE*) *viruses, of the genus* Alphavirus *of the family* Togaviridae*, cause disease ranging from mild febrile reactions to fatal encephalitic zoonoses in Equidae and humans. They are transmitted by haematophagous insects, primarily mammalophilic mosquitoes.*

The VEE complex of viruses includes six antigenic subtypes (*I–VI*)*. Within subtype I there are five antigenic variants* (*variants AB–F*)*. Originally, subtypes I-A and I-B were considered to be distinct variants, but they are now considered to be identical* (*I-AB*)*. Antigenic variants I-AB and I-C are associated with epizootic activity in equids and human epidemics. Historically, severe outbreaks have involved many thousands of human and equine cases. The other three variants of subtype I* (*I-D, I-E, I-F*) *and the other five subtypes of VEE* (*II–VI*) *circulate in natural enzootic cycles. Equidae serve as amplifying hosts for epizootic VEE strains while enzootic VEE viruses cycle primarily between sylvatic rodents and mosquitoes. Enzootic variants and subtypes have been considered to be nonpathogenic for equids, but can cause clinical disease in humans. During 1993 and 1996 ~~however,~~ limited outbreaks of encephalitis in horses in Mexico were shown to be caused by enzootic VEE viruses of subtype I-E. More recently, sporadic outbreaks have occurred in Mexico, Central America, and northern and western parts of South America. Human enzootic subtypes reach more broadly into northern Central America and South America* (*Weaver, 2012*).

Identification of the agent: *Diagnosis of VEE virus infection can be confirmed by the isolation, identification, and antigenic classification of the isolated virus.*

A presumptive diagnosis of equine encephalomyelitis can be made when susceptible animals in tropical or subtropical areas display clinical signs of encephalomyelitis where haematophagous insects are active. VEE virus can be isolated in cell cultures or in laboratory animals using the blood or serum of febrile animals in an early stage of infection. It is recovered less frequently from the blood or brain tissue of encephalitic animals.

VEE virus can be identified by polymerase chain reaction, complement fixation, haemagglutination inhibition, plaque reduction neutralisation (*PRN*), *or immunofluorescence tests using VEE-specific antibodies. Specific identification of epizootic VEE variants can be made by the indirect fluorescent antibody test, or a differential PRN test using subtype- or variant-specific monoclonal antibody, or by nucleic acid sequencing.*

Serological tests: *Specific antibodies may be demonstrated by PRN tests against epizootic VEE virus variants or by IgM capture enzyme-linked immunosorbent assay. Antibody can also be demonstrated by the haemagglutination inhibition or the complement fixation tests.*

Any diagnosis of VEE in an individual that is based on seroconversion in the absence of an epizootic should be made with care. Infections of equids with enzootic VEE viruses produce a low level viraemia accompanied by antibody development, but without clinical disease in most cases. Antibody induced by such subclinical infections may be reactive to epizootic VEE virus variants.

Requirements for vaccines ~~and diagnostic biologicals~~: *The only acceptable vaccines against VEE are an attenuated virus vaccine, made with strain TC-83, or inactivated virus preparations also made from this strain. Attenuated virus is immunogenic when given by intramuscular injection, but sometimes causes adverse reactions in the recipient.*

Formalin-inactivated virulent VEE virus preparations should never be used in equids, as residual virulent virus can remain after formalin treatment, and thereby cause severe illness in both animals and humans. Epizootics of VEE have occurred from the use of such formalin-treated viruses.

A. INTRODUCTION

Venezuelan equine encephalomyelitis (VEE) is an arthropod-borne inflammatory viral infection of equines and humans, resulting in mild to severe febrile and, occasionally fatal, encephalitic disease.

VEE viruses form a complex within the genus *Alphavirus*, family *Togaviridae*. The VEE virus complex is composed of six subtypes (I–VI). Subtype I includes five antigenic variants (AB–F), of which variants 1-AB and 1-C are associated with epizootic VEE in equids and concurrent epidemics in humans (Calisher *et al*., 1980; Monath & Trent, 1981; Pan-American Health Organization, 1972; Walton, 1981; Walton *et al*., 1973; Walton & Grayson, 1989). The epizootic variants 1-AB and 1-C are thought to originate from mutations of the enzootic 1-D serotype (Weaver *et al*., 2004); 1-AB and 1-C isolates have only been obtained during equine epizootics. The enzootic strains include variants 1-D, 1-E and 1-F of subtype I, subtype II, four antigenic variants (A–D) of subtype III, and subtypes IV–VI. Normally, enzootic VEE viruses do not produce clinical encephalomyelitis in the equine species (Walton *et al*., 1973), but in 1993 and 1996 in Mexico, the 1-E enzootic subtype caused limited epizootics in horses. The enzootic variants and subtypes can produce clinical disease in humans (Monath & Trent, 1981; Pan-American Health Organization, 1972; Powers *et al*., 1997; Walton, 1981; Walton & Grayson, 1989).

Historically, epizootic VEE was limited to northern and western South America (Venezuela, Colombia, Ecuador, Peru and Trinidad) (Pan-American Health Organization, 1972). From 1969 to 1972, however, epizootic activity (variant 1-AB) occurred in Guatemala, El Salvador, Nicaragua, Honduras, Costa Rica, Belize, Mexico, and the United States of America (USA) (Texas). Epizootics of VEE caused by I-AB or I-C virus have not occurred in North America and Mexico since 1972. Recent equine and human isolations of epizootic VEE virus were subtype 1-C strains from Venezuela in 1993, 1995 and 1996 and Colombia in 1995.

The foci of enzootic variants and subtypes are found in areas classified as tropical wet forest, i.e. those areas with a high water table or open swampy areas with meandering sunlit streams. These are the areas of the Americas where rainfall is distributed throughout the year or areas permanently supplied with water. Enzootic viruses cycle among rodents, and perhaps birds, by the feeding of mosquitoes (Monath & Trent, 1981; Pan-American Health Organization, 1972; Walton, 1981; Walton & Grayson, 1989). Enzootic VEE strains have been identified in the Florida Everglades (subtype II), Mexico (variant I-E), Central American countries (variant I-E), Panama (variants I-D and I-E), Venezuela (variant I-D), Colombia (variant I-D), Peru (variants 1-D, III-C, and III-D), French Guiana (variant III-B and subtype V), Ecuador (variant I-D), Suriname (variant III-A), Trinidad (variant III-A), Brazil (variants I-F and III-A and subtype IV), and Argentina (subtype VI). In an atypical ecological niche, variant III-B has been isolated in the USA (Colorado and South Dakota) in an unusual association with birds (Monath & Trent, 1981; Pan-American Health Organization, 1972; Walton, 1981; Walton & Grayson, 1989). Everglades virus is a subtype II VEE virus that infects rodents and dogs in Florida.

A tentative diagnosis of viral encephalomyelitis in equids can be based on the occurrence of acute neurological disease during the summer in temperate climates or in the wet season in tropical or subtropical climates. These are the seasons of haematophagous insect activity. Virus infection will result in clinical disease in many equids concurrently rather than in isolated cases. Epizootic activity can move vast distances through susceptible populations in a short time (Monath & Trent, 1981; Pan-American Health Organization, 1972; Walton, 1981; Walton & Grayson, 1989). Differential diagnoses include eastern or western equine encephalomyelitis (chapter 2.5.5), Japanese encephalitis (chapter 2.1.7), West Nile fever (chapter 2.1.20), rabies (chapter 2.1.13), and other infectious, parasitic, or non-infectious agents producing similar signs.

Human VEE virus infections have originated by aerosol transmission from the cage debris of infected laboratory rodents and from laboratory accidents. Infections with both epizootic and enzootic variants and subtypes have been acquired by laboratory workers (American Committee on Arthropod-Borne Viruses [ACAV], 1980). Severe clinical disease or death can occur in humans. Those who handle infectious VEE viruses or their antigens prepared from infected tissues or cell cultures should be vaccinated and shown to have demonstrable immunity in the form of VEE virus-specific neutralising antibody (Berge *et al*., 1961; Pan-American Health Organization, 1972). If vaccination is not a viable option, additional personal protective equipment to include respiratory protection is recommended for all procedures. ~~All procedures producing aerosols from VEE virus materials should be conducted in biosafety cabinets at containment level 3 (see Chapter 1.1.2 *Biosafety and biosecurity in the veterinary microbiological laboratory and animal facilities*) (ACAV, 1980; United States Department of Health and Human Services, 2009 1999).~~ Laboratory manipulations should be carried out at an appropriate biosafety and containment level determined by biorisk analysis (see Chapter 1.1.3 *Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities*).

B. DIAGNOSTIC TECHNIQUES

Table 1. *Test methods available for the diagnosis of Venezuelan equine encephalomyelitis and their purpose*

Method	Purpose				
	Population freedom from infection	Individual animal freedom from infection	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Agent Identification	–	+	+++	–	–
IgM Capture ELISA	–	–	++	–	–
Plaque reduction neutralisation (paired samples)	+++	+	++	++	+++
Hemagglutination inhibition (paired samples)	+	++	++	++	++
Complement fixation (paired samples)	–	+	++	–	–

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose. although not all of the tests listed as category +++ or ++ have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.
ELISA = enzyme-linked immunosorbent assay.

1. Identification of the agent

A confirmatory diagnosis of VEE is based on the isolation and identification of the virus or on the demonstration of seroconversion. The period of viraemia coincides with the onset of pyrexia within 12–24 hours of infection. Viraemia terminates 5–6 days after infection, and coincides with the production of neutralising antibodies and the appearance of clinical neurological signs. Frequently, VEE viruses cannot be isolated from the brains of infected equids. Blood samples for virus isolation should be collected from febrile animals that are closely associated with clinical encephalitic cases.

Virus may be isolated from the blood or sera of infected animals by inoculating 1–4-day-old mice or hamsters intracerebrally or by the inoculation of other laboratory animals, such as guinea-pigs and weaned mice. It may also be isolated by the inoculation of various cell cultures including African green monkey kidney (Vero), rabbit kidney (RK-13), baby hamster kidney (BHK-21), or duck or chicken embryo fibroblasts, or by inoculation of embryonated chicken eggs. Details of virus identification techniques are described in chapter 2.5.5.

Isolates can be identified as VEE virus by reverse transcriptase-polymerase chain reaction (RT-PCR), complement fixation (CF), haemagglutination inhibition (HI), or plaque reduction neutralisation (PRN) tests, or by immunofluorescence as described in chapter 2.5.5. The VEE virus isolates can be characterised by the indirect fluorescent antibody or PRN tests using monoclonal antibody or by nucleic acid sequencing. The VEE virus characterisation should be carried out in a reference laboratory (see Table given in Part 4 of this *Terrestrial Manual*).

2. Serological tests

Diagnosis of VEE virus infection in equids requires the demonstration of specific antibodies in paired serum samples collected in the acute and convalescent phases. After infection, PRN antibodies appear within 5–7 days, CF antibodies within 6–9 days, and HI antibodies within 6–7 days. The second convalescent phase serum sample should be collected 4–7 days after the collection of the first acute phase sample or at the time of death. The serological procedures are described in detail in chapter 2.5.5. Vaccination history must be taken into account when interpreting any of the VEE serological test results. In horses not recently vaccinated with an attenuated live virus strain, demonstration of VEE-specific serum IgM antibodies in a single serum sample supports recent virus exposure.

Any diagnosis of VEE in an individual that is based on seroconversion in the absence of an epizootic should be made with care. Although enzootic subtypes and variants are nonpathogenic for equids, infection will stimulate antibody production to epizootic VEE virus variants.

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

1. Background

The acceptable vaccines against VEE infection are an attenuated virus vaccine, strain TC-83, and an inactivated virus preparation made from that strain (Monath & Trent, 1981; Pan-American Health Organization, 1972; Walton, 1981; Walton & Grayson, 1989). ~~The inactivated vaccine is now the most widely used, and is marketed in EEE/VEE, EEE/WEE/VEE, EEE/WEE//VEE/tetanus toxoid, and EEE/WEE/VEE/West Nile virus/tetanus toxoid combinations.~~ Directions for use provided with commercial products should be followed; examples of typical guidelines are below.

Inactivated vaccine should be administered in two doses with an interval of 2–4 weeks between doses. Annual revaccination is recommended.

Attenuated vaccine should be reconstituted with physiological saline and used immediately. Multidose vials are kept on ice while the vaccine is being used. Any vaccine not used within 4 hours ~~1 hour~~ of reconstitution should be safely discarded. ~~Foals under 2 weeks of age and pregnant mares should not be vaccinated.~~ Animals over 3 months of age are vaccinated subcutaneously in the cervical region with a single dose. Annual revaccination is ~~not~~ recommended.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 on *Principles of veterinary vaccine production*. The guidelines given below and in chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements.

~~NOTE: Formalin treated preparations of virulent epizootic VEE virus should never be used in equids. Residual virulent virus can remain after formalin treatment, and result in severe illness. Epizootics of VEE have occurred in Central and Southern America from the use of such preparations (Walton, 1981; Weaver et al., 1999).~~

~~1. Seed management~~[1]

2. Outline of production and minimum requirements for vaccines

a) Characteristics of the seed

See chapter 1.1.6 *Principles of veterinary vaccine production*, for general requirements for master seeds and allowable passages for vaccine production. Suitable seed lots should be maintained at –70°C in a lyophilised state.

i) *Biological characteristics of the master seed*

~~Attenuated~~ The VEE virus vaccine strain TC-83 originated from the Trinidad donkey strain (a variant of I-AB) of epizootic VEE virus isolated in 1944. This strain was derived by serial passage of the Trinidad donkey strain in fetal guinea-pig heart cells. It is safe and immunogenic at the established passage levels, and induces protective immunity in vaccinated equids, although adverse reactions can sometimes occur. The vaccine was originally developed for use in personnel involved in high-risk VEE virus research. ~~Suitable seed lots should be maintained at –70°C in a lyophilised state.~~

~~b) Method of culture~~

~~The virus is grown in fetal guinea pig heart cell cultures in a suitable medium.~~

~~c) Validation as a vaccine~~

~~The cells used for vaccine production must be free from bacterial, fungal, mycoplasmal, and viral contamination. VEE virus is identified in batches of vaccine by PRN tests against hyperimmune serum. For inactivated vaccines of cell culture origin, strain TC-83 virus is treated with formaldehyde.~~

~~1 Sections on Seed management, Manufacture, In-process control, and Batch control are taken from the Biotechnology, Biologics, and Environmental Protection Division of the United States Department of Agriculture's Animal and Plant Health Inspection Service (APHIS).~~

~~**2. Method of manufacture (see footnote 1)**~~

~~Vaccine is produced by harvesting supernatant fluid from fetal guinea-pig heart monolayers in which the replication of attenuated VEE virus has occurred. The monolayers are maintained at approximately 37°C. The time of harvesting is determined by the occurrence of characteristic cytopathic changes when approximately 70–100% of the cell sheet is affected, typically 1–3 days after infection. The supernatant fluid is clarified by low speed centrifugation and suitable stabilisers are added to protect the virus during freezing and lyophilisation.~~

ii) *Quality criteria* (*sterility, purity, freedom from extraneous agents*)

The MSV must be tested for purity, identity, and freedom from extraneous agents at the time before it is used in the manufacture of vaccine. The MSV must be free from bacteria, fungi and mycoplasma. The MSV is cultured on a Vero cell line and an embryonic equine cell type with confirmation by the fluorescent antibody technique to demonstrate freedom from equine herpesvirus, equine adenovirus, equine arteritis virus, bovine viral diarrhoea virus, reovirus, and rabies virus extraneous agents The MSV must also be free from extraneous virus by cytopathic effect (CPE) and haemadsorption on cell culture.

iii) *Validation as a vaccine strain*

In an immunogenicity trial, the MSV at the highest passage level intended for production must prove its efficacy (protection) in the guinea-pig vaccination/serology potency test.

iv) *Emergency procedure for provisional acceptance of new master seed virus* (*MSV*) *in the case of an epizootic* (*with pathogens with many serotypes, e.g. bluetongue virus, highly pathogenic avian influenza, FMD, etc*.)

In emergency epizootic situations, provisional acceptance of a new strain could be based on a risk analysis of the possibility of contamination of the antigen produced from the new MSV with extraneous agents. This risk assessment should take into account the characteristics of the process, including the nature and concentration of the inactivant for inactivated vaccines, before allowing or not the early release of the new product However, formalin-treated preparations of virulent epizootic VEE virus should never be used in equids. Residual virulent virus can remain after formalin treatment, and result in severe illness. Epizootics of VEE have occurred in Central and Southern America from the use of such preparations (Walton, 1981; Weaver *et al.,* 1999).

b) Method of manufacture

i) *Procedure*

The MSV should be propagated in cell lines known to support the growth of VEE. See chapter 1.1.6 for additional guidance on the preparation and testing of master cell stocks. Cell lines should be free from extraneous viruses, bacteria, fungi, and mycoplasma. Viral propagation should not exceed five passages from the MSV, unless further passages prove to provide protection in the host animal.

The susceptible cell line is seeded into suitable vessels. Minimal essential medium, supplemented with fetal bovine serum (FBS), may be used as the medium for production. Incubation is at 37°C.

Cell cultures are inoculated directly with VEE working virus stock, which is generally 1 to 4 passages from the MSV. Inoculated cultures are incubated for 1–3 days before harvesting the culture medium. During incubation, the cultures are observed daily for CPE and bacterial contamination.

The TC-83 VEE vaccine strain may be chemically inactivated with formalin and mixed with a suitable adjuvant. The duration of the inactivation period is based on demonstrated inactivation kinetics.

The preservatives used are thimerosal at a 1/1000 dilution and antibiotics (neomycin, polymyxin, amphotercin B, and gentamicin).

ii) *Requirements for ingredients*

All ingredients used in the manufacture of VEE vaccine should be defined in approved manufacturing protocols and consistent from batch to batch. See chapter 1.1.6 for general guidance on ingredients of animal origin. Ingredients of animal origin should be sourced from a country with negligible risk for transmissible spongiform encephalopathies (TSEs).

iii) *In-process controls*

Production lots should be examined daily for cytopathic changes. After harvesting, the virus suspension should be tested for the presence of microbial contaminants. Production lots of VEE must be titrated in tissue culture before inactivation to standardise the product. Low-titred lots may be concentrated or blended with higher-titred lots to achieve the correct titre.

Inactivated VEE lots must be tested for completeness of inactivation in 6- to 12-hour old chicks.

iv) *Final product batch tests*

Sterility

Inactivated and live vaccine samples are examined for bacterial and fungal contamination. The volume of medium used in these tests should be enough to nullify any bacteriostatic or fungistatic effects of the preservatives in the product. To test for bacteria, ten vessels, each containing a minimum of 120 ml of soybean casein digest medium, are inoculated with 0.2 ml from ten final-container samples. The ten vessels are incubated at 30–35°C for 14 days and observed for bacterial growth. To test for fungi, ten vessels, each containing a minimum of 40 ml of soybean casein digest medium, are inoculated with 0.2 ml from ten final-container samples. The vessels are incubated at 20–25°C for 14 days and observed for fungal growth. Individual countries may have other requirements.

Identity

Separate batch tests for identity should be conducted if the batch potency test, such as tissue-culture titrations of live virus vaccines, does not sufficiently verify the identity of the agent in the vaccine. Identity tests may include fluorescent antibody or serum neutralisation assays.

Safety

Batch safety testing for VEE inactivation is conducted in 6- to 12-hour old chicks. Ten chicks are inoculated subcutaneously with 0.5 ml of product and observed daily for 10 days. If unfavourable reactions occur, the batch is unacceptable.

Batch potency

Potency tests of the inactivated vaccine are described in chapter 2.5.5 except that the acceptable antibody titre in inoculated guinea-pigs will be ≥1/4.

c) Requirements for authorisation/registration/licensing

i) *Manufacturing process*

For registration of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Section C.2.a and b) should be submitted to the authorities. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

In-process controls are part of the manufacturing process.

ii) *Safety requirements*

The final inactivated vaccine formulation should be tested in a limited number of target animals prior to a larger-scale field study. The final vaccine formulation should not cause adverse reactions.

Field safety studies should be conducted before any vaccine receives final approval. Generally, two serials should be used, in three different geographical locations under typical animal husbandry conditions, and in a minimum of 600 animals. The vaccine should be administered according to label recommendations (including booster doses) and should contain the maximum permissible amount of VEE antigen. (If no maximum antigen content is specified, serials should be of anticipated typical post-marketing potency.) About one-third of the animals should be at the minimum age recommended for vaccination.

- *Precautions* (*hazards*)

  Vaccine should be identified as harmless or pathogenic for vaccinators. Manufacturers should provide adequate warnings that medical advice should be sought in the case of self-injection (including for adjuvants, oil-emulsion vaccine, preservatives, etc.) with warnings included on the product label/leaflet so that the vaccinator is aware of any danger.

iii) *Efficacy requirements*

To register a commercial vaccine, a batch or batches produced according to the standard method and containing the minimum amount of antigen or potency value shall prove its efficacy (protection) in the guinea-pig vaccination/serology potency test; each future commercial batch shall be tested before release to ensure it has the same potency value demonstrated by the batch(es) used for the efficacy test(s).

iv) *Vaccines permitting a DIVA strategy* (*detection of infection in vaccinated animals*)

Vaccination is only recommended for horses in VEE-positive areas. Vaccinated horses may develop a serological titre, which may interfere with the ability to export the horse.

3. In-process control (see footnote 1)

Cultures should be examined daily for cytopathic changes. After harvesting, the virus suspension should be tested for the presence of microbial contaminants.

Inactivated vaccines derived from attenuated strain TC-83 virus should be checked to exclude the presence of viable virus after formalin treatment.

4. Batch control (see footnote 1)

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.9.

b) Safety

Safety tests of the inactivated vaccine are described in Chapter 2.5.5.

Safety tests of the attenuated vaccine are conducted in mice. A 0.5 ml dose is injected intraperitoneally or subcutaneously into each of eight mice, and the animals are kept under observation for 7 days. If adverse reactions attributable to the product occur during this period, the product is considered to be unsatisfactory.

c) Potency

Potency tests of the inactivated vaccine are described in Chapter 2.5.5 except that antibody titre in inoculated guinea-pigs will be ≥1/4.

Potency of the attenuated vaccine can be determined by testing in horses. Each of 20 susceptible horses is inoculated subcutaneously with 1 ml of lyophilised vaccine that has a reconstituted virus titre of at least 2.5 $\log_{10}$ $TCID_{50}$ (50% tissue culture infective dose) per ml. For a valid test, at least 19 of 20 vaccinated horses must have HI antibody titres of at least 1/20 or serum neutralising antibody titres of at least 1/40 within 21–28 days of vaccination.

When tested at any time within the expiration period following lyophilisation, the product must have a virus titre of 0.7 $\log_{10}$ greater than that used to test horses as described above, but no less than 2.5 $\log_{10}$ $TCID_{50}$/dose.

The final product must be free from bacterial, fungal, mycoplasmal, or extraneous viral contaminants.

d) Duration of immunity

v) *Duration of immunity*

Comprehensive studies on duration of immunity are not available. An annual revaccination is recommended for the inactivated vaccine. Foals that are vaccinated at under 1 year of age should be revaccinated before the next vector season. Revaccination with the attenuated vaccine is not recommended.

e) Stability

v) *Stability*

The lyophilised inactivated vaccine is stable and immunogenic for 2 3 years if kept refrigerated at 2–7°C. After 2 3 years, vaccine should be discarded. The vaccines should be used immediately after reconstitution. Multidose vials of the attenuated vaccine should be kept on ice while being used. All unused vaccine should be safely discarded 4 hours after reconstitution.

f) Preservatives

The preservatives used are thimerosal at a 1/1000 dilution and antibiotics (neomycin, polymyxin, amphotercin B, and gentamicin).

g) Precautions (hazards)

Pregnant mares and foals under 2 weeks of age should not be vaccinated.

All personnel handling infectious VEE viruses or their antigens prepared from infected tissues or cell cultures should be vaccinated and shown to have demonstrable immunity in the form of VEE virus-specific

~~neutralising antibody. All procedures producing aerosols from VEE virus materials should be conducted in biosafety cabinets with biocontainment and efficient filtration of the exhaust air from the laboratory (ACAV, 1980; United States Department of Health and Human Services, 1999).~~

~~5. Tests on the final product~~

~~a) Safety and potency~~

~~Safety and potency tests are as outlined above under Batch control (Sections C.4.b and C.4.c). The attenuated vaccine must have a virus titre of no less than 2.5 $\log_{10}$ $TCID_{50}$/dose.~~

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* *

NB: There is an OIE Reference Laboratory for Venezuelan equine encephalomyelitis
(see Table in Part 4 of this *Terrestrial Manual* or consult the OIE web site for the most up-to-date list:
http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/ http://www.oie.int/).
Please contact the OIE Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for Venezuelan equine encephalomyelitis

CHAPTER 2.7.5.

CONTAGIOUS AGALACTIA

SUMMARY

Contagious agalactia is a serious disease syndrome of sheep and goats that is characterised by mastitis, arthritis, keratoconjunctivitis and, occasionally, abortion. Mycoplasma agalactiae (Ma) *is the main cause of the disease in sheep and goats, but* M. capricolum *subsp.* capricolum (Mcc), M. mycoides *subsp.* capri (Mmc) ~~(*formerly named* M. mycoides *subsp.* mycoides *LC* [*LC = large colonies*])~~ *and* M. putrefaciens *produce a clinically similar disease, more often in goats, which may be accompanied by pneumonia.* Ma *and* Mcc *have been isolated from wild small ruminants such as ibex and mountain goats. Antibodies to* Mmc *and* Mcc *have been detected in South American camelids* (*alpacas, llamas and vicunas*), *but no mycoplasmas have yet been isolated.*

Identification of the agent: *Definitive diagnosis requires the isolation of the causative mycoplasmas from the affected animals, which are then identified by biochemical, serological and, increasingly, molecular tests such as the polymerase chain reaction. Samples of choice include milk, conjunctival and ear swabs, and joint fluid. The sampling of bulk milk tank provides a convenient way of monitoring whole herds/flocks for causative mycoplasmas. All four mycoplasmas grow relatively well in most mycoplasma media although* M. agalactiae *shows a preference for organic acids such as pyruvate as substrates.*

Serological tests: *Detection of antibodies in serum by ~~complement fixation test or~~ enzyme-linked immunosorbent assay* (*ELISA*) *provides rapid diagnosis of disease, but may not be very sensitive in chronically affected herds and flocks. Indirect ELISAs have been used routinely in control programmes for screening herds for* Ma~~. agalactiae~~. *Confirmation of infection by isolation and identification or detection by polymerase chain reaction is usually necessary in areas believed to be free of contagious agalactia. Serological tests are not widely available for* M. putrefaciens.

Requirements for vaccines ~~and diagnostic biologicals~~: *Commercial vaccines for* Ma~~. agalactiae~~, *inactivated with formalin, are widely used in southern Europe, but are not considered to be very efficacious. Under experimental conditions,* Ma~~. agalactiae~~ *vaccines inactivated with saponin ~~or phenol~~ have been shown to be more protective than formalised preparations. Live vaccines for* Ma~~. agalactiae~~ *are used in Turkey, where they are reported to be more protective than inactivated vaccines. A commercial vaccine containing* Ma~~. agalactiae~~, Mmc *and* Mcc *is available. Autogenous vaccines for* Mmc *and, occasionally, for* Mcc *are believed to be used in some countries. No vaccines exist* for M. putrefaciens*, as the disease it causes is not considered to be sufficiently serious or widespread.*

A. INTRODUCTION

Contagious agalactia is a disease of sheep and goats that is characterised by mastitis, arthritis and keratoconjunctivitis, and has been known for nearly 200 years. It occurs in Europe, ~~western~~ Asia, the United States of America (USA) and North Africa, and is mainly caused by *Mycoplasma agalactiae* (*Ma*) (reviewed by Bergonier *et al*., 1997). An upsurge of contagious agalactia, caused by *Ma*, has been seen in sheep in Spain and France recently with increased cases reported in and around the Pyrenees and new outbreaks being reported in Corsica (Chazel *et al*., 2010). Frequent and numerous outbreaks occur in Iran and Mongolia (Nicholas *et al*., 2008). In recent years, *M. capricolum* subsp. *capricolum* (*Mcc*) and *M. mycoides* subsp. *capri*[1] (formerly *M. mycoides* subsp. *mycoides* LC [LC = large colonies]) have also been isolated from sheep and goats with

1 ~~International Committee on Systematics of Prokaryotes Subcommittee on the Taxonomy of Mollicutes has proposed the merging of these two subspecies into the single subsp: *M. mycoides* subsp. *capri;* a decision is pending.~~

mastitis and arthritis in many countries, including those in South America (Nascimento *et al,* 1986) and Australasia (Cottew 1971).

The clinical signs of ~~these~~ infections caused by *Mcc, Mmc* and *Mp* are sufficiently similar to be considered indistinguishable from contagious agalactia caused by *Ma*. In addition, *M. putrefaciens* (*Mp*) also causes mastitis and arthritis in goats, which is very similar to that caused by ~~*M. agalactiae*~~ *Ma*, *Mmc* and *Mcc* (Rodriguez *et al*., 1994). Furthermore, the consensus of the working group on contagious agalactia of the EC COST[2] Action 826 on ruminant mycoplasmoses, which met in Toulouse, France, in 1999, was that all four mycoplasmas should be considered as causal agents of contagious agalactia. In France, *Mmc*, *Mcc* and *Mp* constitute over 80% of the mycoplasma isolations from goats with *Ma* accounting for less than 2%. *Ma* and *Mcc* have been isolated from wild small ruminants such as ibex and mountain goats in the Pyrenees and Alps (Chazel *et al*., 2010; Verbsick *et al*., 2008). There are occasional reports of the isolation of *Ma* from apparently healthy cattle (Chazel *et al.,* 2010).

Clinically, the disease caused by ~~*M. agalactiae*~~ *Ma* is recognised by elevated temperature, inappetence and alteration in the consistency of the milk in lactating ewes with decline and subsequent failure of milk production, often within 2–3 days, as a result of interstitial mastitis (Bergonier *et al*., 1997); lameness and keratoconjunctivitis affects about 5–10% of infected animals. Fever is common in acute cases and may be accompanied by nervous signs, but these are rare in the more frequently observed subacute and chronic infections. Pregnant animals may abort. ~~*Mycoplasma agalactiae*~~ *Ma* may occasionally be found in lung lesions (Loria *et al*., 1999), but pneumonia is not a consistent finding. Bacteraemia is common, particularly for *Mmc* and *Mcc* and could account for the isolation of the organism from sites where it is only present transiently.

Mastitis, arthritis, pleurisy, pneumonia, and keratoconjunctivitis may all result from infection with *Mmc,* which has one of the widest geographical distribution of ruminant mycoplasmas, being found on all continents where small ruminants are kept and wherever contagious agalactia and caprine pleuropneumonia are reported (DaMassa *et al*., 1983; Nicholas, 2002); however the lack of diagnostic facilities for mycoplasma diseases in many countries means that it is probably under reported. *Mmc* is mostly confined to goats but has occasionally been isolated from sheep with reproductive disease and cattle with arthritis or respiratory disease. Cases usually occur sporadically, but the disease may persist and spread slowly within a herd. After parturition, the opportunity for spread in milking animals increases, and kids ingesting infected colostrum and milk become infected. The resulting septicaemia, with arthritis and pneumonia, causes high mortality in kids (Bergonier *et al*., 1997; DaMassa *et al*., 1983).

Mcc is widely distributed and highly pathogenic, particularly in North Africa, but the frequency of occurrence is low (Bergonier *et al*., 1997). Goats are more commonly affected than sheep, and clinical signs of fever, septicaemia, mastitis, and severe arthritis may be followed rapidly by death (Bergonier *et al*., 1997; Bolske *et al*., 1988). Pneumonia may be seen at necropsy. The severe joint lesions seen in experimental infections with this organism are accompanied by intense periarticular subcutaneous oedema affecting tissues some distance from the joint (Bolske *et al*., 1988).

~~*Mycoplasma putrefaciens*~~ *Mp* is common in milking goat herds in western France where it can be isolated from animals with and without clinical signs (Mercier *et al*., 2001). It has also been associated with a large outbreak of mastitis and agalactia leading to severe arthritis in goats accompanied by abortion and death without pyrexia in California, USA (Bergonier *et al*., 1997). ~~*Mycoplasma putrefaciens*~~ *Mp* was the major finding in an outbreak of polyarthritis in kids in Spain (Rodriguez *et al*., 1994).

Antibodies to *Mmc* and *Mcc*, but not ~~*M. agalactiae*~~ *Ma*, have been detected in South American camelids, including llamas, alpacas and vicunas, but as yet no mycoplasmas have been isolated (Nicholas, 1998). These camelids are affected by a range of mycoplasma-like diseases, including polyarthritis and pneumonia, so it is likely that mycoplasmas including *Mmc* and *Mcc* may be found in the future.

1 European Cooperation in the field of Scientific and Technical Research.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection	Efficiency of eradication policies	Confirmation of clinical cases	Prevalence of infection - surveillance	Immune status in individual animals or populations post-vaccination
Culture and identification of the organism	++	+++	+++	+++	-	-
Complement fixation	+++*	+	+++	+++*	+++*	+++*
Enzyme-linked immunosorbent assay	+++**	+	+++	+++**	+++**	+++**
Polymerase chain reaction	++	+++	-	+++	++	-
Immunoblotting	+	++	+	++	+	++

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose.
Although not all of the tests listed as category +++ or ++ have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.
* for Mcc and Mmc only; ** for Ma only

1. Identification of the agents

a) Selection of samples

Preferred samples from living animals include: nasal swabs and secretions; milk from mastitic females or from apparently healthy females where there is a high rate of mortality/morbidity in kids; joint fluid from arthritic cases; conjunctival swabs from cases of ocular disease; and blood for antibody detection from affected and non-affected animals (Nicholas & Baker, 1998). The sampling of bulk milk tank provides a convenient way of monitoring flocks and herds for causative mycoplasmas. A distinctive smell of putrefaction in the milk is often the first sign of the presence of *Mp* infection in a herd. The ear canal has also been shown to be a source of pathogenic mycoplasmas, although in practice the presence of nonpathogenic mycoplasmas at this site may make confirmation difficult (Nicholas & Baker, 1998). Mycoplasmas may be isolated from the blood during the acute stage of the disease when there is mycoplasmaemia. From dead animals, samples should include: udder and associated lymph nodes, joint fluid, lung tissue (at the interface between diseased and healthy tissue) and pleural/pericardial fluid. Samples should be dispatched quickly to a diagnostic laboratory in a moist and cool condition. All four causative mycoplasmas are relatively easy to isolate from internal organs, joints and milk and grow well in most mycoplasma media, producing medium to large colonies in 3–4 days.

b) *Mycoplasma* ~~medium~~ isolation

The usual techniques used in the isolation of mycoplasmas apply to all four causative organisms (Nicholas & Baker, 1998). Many media have been reported to grow the causative mycoplasmas. Improved growth rates of ~~*M. agalactiae*~~ *Ma* have been seen in media containing organic acids such as pyruvate and isopropanol (Khan *et al*., 2004). The formulation of PRM medium (Khan *et al*., 2004) is as follows:

Heat inactivated porcine serum 100 ml/litre, special peptone 20 g/litre, yeast extract 5 g/litre, glycerol 5 g/litre, sodium chloride 5 g/litre, HEPES 9 g/litre, fresh yeast extract 100 ml/litre, sodium pyruvate 5 g/litre, 12.5 ml of 0.2% phenol red and ampicillin (200,000 International Units/ml. Make up to 1 litre in distilled water and sterilise by filtration. Adjust the pH of the broth medium to 7.6. Prepare solid medium by adding 10 g of LabM agar No. 1 (Bury UK, or agar of equivalent quality) and dispense into sterile Petri dishes.

Thallium acetate (250 mg/litre), which is toxic and inhibitory to some mycoplasmas but not those causing contagious agalactia, may be a necessary component of the transport medium to reduce bacterial contamination from clinical samples, but should be omitted once the mycoplasmas begin to grow *in vitro*. A satisfactory alternative to thallium acetate may be colistine sulphate (37.5 mg/litre).

- **Test procedure**

i) Make tenfold dilutions (10^1–10^6) of the liquid sample (milk, synovial fluid, conjunctival and ear swabs) or tissue homogenate in appropriate broth medium.

ii) Spread a few drops of each sample on the agar medium and dispense a 10% (v/v) inoculum into broth medium.

iii) Streak swabs directly on to agar medium.

iv) Incubate inoculated broths (optimally with gentle shaking) and agar media at 37°C in humidified atmosphere with 5% carbon dioxide.

v) Examine broths daily for signs of growth (indicated by a fine cloudiness or opalescence) or changes in pH indicated by a colour change and examine agar media under × 35 magnification for typical 'fried egg' colonies.

vi) If no mycoplasma growth is seen after 7 days, subculture a 10% (v/v) inoculum of broth into fresh broth and spread about 50 µl of this on to agar media.

vii) Repeat as for step v. If no mycoplasmas are seen after 21 days' incubation, consider the results to be negative.

viii) If bacterial contamination results (seen as excessive turbidity), filter sterilise by passing 1 ml of contaminated broth through a 0.45 µm filter into fresh broth medium.

Clinical samples frequently contain more than one mycoplasma species so clone purification of colonies is often considered necessary before performing biochemical and serological identification, in particular the growth and film inhibition tests (GIT and FIT, respectively). However, cloning is a lengthy procedure taking at least 2 weeks. The immunofluorescence test (Bradbury, 1998), dot immunobinding tests (Poumarat, 1998) and, more recently, polymerase chain reaction (PCR) tests (see Section B.1.e) do not require cloning as these tests can detect the pathogenic mycoplasmas in mixed cultures, saving a great deal of time.

c) Biochemical tests

The first test that should be performed on the cloned isolates is sensitivity to digitonin, which separates mycoplasmas from acholeplasmas; the latter are ubiquitous contaminants that can overgrow the mycoplasmas of interest. Growth in liquid medium containing glucose (1%), arginine (0.2%), and phenolphthalein diphosphate (0.01%), on solid medium containing horse serum or egg yolk for the demonstration of film and spots, and on casein agar or coagulated serum agar to test for proteolysis, are among the most useful tests for differentiating the four mycoplasmas (Poveda, 1998). These biochemical characteristics, however have been increasingly found to be variable for the individual mycoplasmas and have little diagnostic value. The most impressive biochemical characteristic that differentiates ~~*M. putrefaciens*~~ *Mp* from all other mycoplasmas is the odour of putrefaction it produces in broth culture. Other features that may be helpful include: film and spot production seen on the surface of the broth and solid media caused by ~~*M. agalactiae*~~ *Ma* and to a lesser extent by ~~*M. putrefaciens*~~ *Mp*; and the proteolytic activity of *Mcc* and *Mmm*LC on casein and coagulated serum.

A rapid and highly convenient biochemical test that exploits the C8-esterase activity of ~~*M. agalactiae*~~ *Ma* has been reported (Khan *et al*., 2001). The mycoplasma forms red colonies on agar media within 1 hour of adding the chromogenic substrate, SLPA-octanoate (a newly synthesised ester formed from a C8 fatty acid and a phenolic chromophore). This activity is shared with *M. bovis,* although this mycoplasma is rarely found in small ruminants. ~~Isolates need not be cloned as *M. agalactiae* can be detected easily in mixed cultures.~~ If necessary PCRs can be used to distinguish rapidly ~~*M. agalactiae*~~ *Ma* from *M. bovis* (see Section B.1.e).

d) Serological identification

Identification of isolates using specific antisera is usually carried out with the GIT, FIT (Poveda & Nicholas, 1998) or the indirect fluorescent antibody (IFA) test (Bradbury, 1998). A ~~recently developed~~ dot immunobinding test, which is carried out in microtitre plates, offers many improvements over the other serological tests such as rapidity and higher throughputs (Poumarat, 1998) but requires subjective judgements of staining intensity. For ~~*M. agalactiae*~~ *Ma*, film inhibition may often be more reliable as growth inhibition is not seen with all isolates; it can also be used for serodiagnosis. Film production by the mycoplasma may be enhanced by the incorporation of 10% egg yolk suspension into the solid medium.

- **Test procedure**

i) Inoculate at least two dilutions of 48-hour cloned broth cultures (10^{-1} and 10^{-2}) on to predried agar media by allowing 50 µl of the cultures to run down the tilted plates using the 'running drop' technique (Poveda & Nicholas, 1998). Remove any excess liquid with a pipette.

ii) Allow the plates to dry. It is possible to apply two or three well separated running drops to each 90 mm diameter plate.

iii) Apply predried filter paper discs containing 30 µl of specific antiserum to the culture; ensure good separation of discs (at least 30 mm).

iv) Incubate the plates as for mycoplasma culture and examine daily by eye against a light background.

- **Interpretation of the results**

A zone of inhibition over 2 mm, measured from the paper disc to the edge of mycoplasma growth is considered to be significant. Partial inhibition can occur with weak antiserum or where there are mixed cultures. Stronger reactions can be obtained if about 60 µl of antisera is added to 6 mm diameter wells made in the agar with a cork borer or similar device (Poveda & Nicholas, 1998).

In the IFA test, specific antisera are applied to colonies on solid medium. Homologous antiserum remains attached after washing and is demonstrated by adding fluorescein-conjugated antiglobulin, washing, and viewing the colonies with an epifluorescence microscope (Bradbury, 1998). Controls should include known positive and known negative control organisms, and a negative control serum. However like the immunobinding tests subjective judgements are required to assess staining intensity.

Antisera for these serological tests have traditionally been prepared against the type strains of the various *Mycoplasma* species, and most field isolates have been readily identified using these antisera. As more strains have been examined, however, some have been found to react poorly with these antisera, while reacting well with antisera to other representative strains of the species. Intraspecies variation in antigenic composition has not been reported for ~~*M. putrefaciens*~~ *Mp*, but occurs to some degree with ~~*M. agalactiae*~~ *Ma* and with *Mcc* strains. Thus, diagnostic laboratories may need to have several antisera to enable all strains of the species to be identified.

e) Nucleic acid recognition methods

i) *Polymerase chain reaction assays*

PCR assays are routinely used in many laboratories and are extremely sensitive. They can provide a rapid early warning system when carried out on clinical samples, enabling a full investigation to take place when results are positive. However negative results should not be considered definitive. Several PCRs specific for ~~*M. agalactiae*~~ *Ma* have been developed and show similar levels of sensitivity, although they are based on different gene sequences (~~Bashiruddin *et al*., 2005;~~ Dedieu *et al*., 1995; Subrahamaniam *et al*., 1998; Tola *et al*., 1997a). They can be used directly on nasal, conjunctival, synovial and tissue samples; they have been used on milk samples where they have been reported to be more sensitive than culture (Tola *et al*., 1997a), although occasionally undefined inhibitors may interfere with the test. PCRs can also be used, more reliably, on mycoplasmas growing in culture; a 24 hour enrichment of the mycoplasma in the appropriate medium greatly facilitates PCR detection even in the presence of bacterial contamination (Nicholas, 2002). A newly described PCR based method called denaturing gradient gel electrophoresis (DGGE) that uses mycoplasma-specific primers is capable of identifying the majority of small ruminant mycoplasmas including all the causative agents of contagious agalactia by their migration pattern (McAuliffe *et al*., 2005). A positive PCR result, particularly in an area previously free of contagious agalactia, should be confirmed by isolation and identification of the mycoplasma using standard procedures.

Individual PCRs have been reported for *Mmc* (Bashiruddin, 1998) and *Mcc* (Monnerat *et al.*, 1999) ~~Bashiruddin *et al*., 1994~~and ~~*M. putrefaciens*~~ *Mp* (Peyraud *et al*., 2003; Nicholas *et al.*, 2008) respectively. In addition a multiplex test has been described that can detect simultaneously ~~*M. agalactiae*~~ *Ma*, *Mcc* and *Mmc* (Greco *et al*., 2001).

ii) *Real-time PCRs*

Several rapid real time PCRs have been reported for *Ma* which provide advantages of speed, sensitivity and sample handling (Lorusso *et al*., 2007). More recently a multiplex real time test has been described which detects all four mycoplasmas simultaneously (Becker *et al*., 2012).

iii) *Micro-array analysis*

Micro-array analysis has been applied to the detection of mycoplasmas. Using probes derived from the 23 rRNA genes and *tuf* gene target regions, Schnee *et al.* (2012) have described the identification of 37 mycoplasma species including all four contagious agalactia pathogens. At the time of publication

some cross reaction was seen between *Ma* and the closely related bovine pathogen, *M. bovis*. The advantages of the test over PCR include ease of operation, high information content and cost-effectiveness.

- **Test procedure**

The following primers based on the uvrC gene have been shown to be specific for ~~*M. agalactiae*~~ *Ma* (Subrahamaniam *et al*., 1998). PCRs may need to be optimised in each laboratory. Positive and negative control DNA should be run in each assay.

MAGAUVRC1-L CTC-AAA-AAT-ACA-TCA-ACA-AGC
MAGAUVRC1-R CTT-CAA-CTG-ATG-CAT-CAT-AA

i) Extract DNA from *Mycoplasma* isolates or clinical material using the appropriate method (Bolske *et al*., 1988).

ii) Carry out PCR methods in 50 µl reaction mixtures containing: 1 µl of sample DNA, 20 pmol of each primer (see above), 1 mM each dNTP, 10 mM Tris/HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl and 1.25 mM U *Taq* DNA polymerase.

iii) Subject the mixture to 35 amplification cycles in a thermal cycler with the following parameters: 30 seconds at 94°C, 30 seconds at 50°C annealing temperature and 1 minute at 72°C.

iv) Analyse the PCR products by electrophoresis on a 0.7% agarose at 110 V for 2 hours and visualise by staining with ethidium bromide. A 1.7 kb fragment indicates the presence of ~~*M. agalactiae*~~ *Ma*.

2. Serological tests

a) Enzyme-linked immunosorbent assay

ELISAs using sonicated or Tween-20-treated antigens have been reported to be more sensitive than the CFT for the detection of antibody to *M. agalactiae* (Bergonier *et al*., 1997). Problems of nonspecificity have been overcome by the use of monoclonal or protein G conjugates in the ELISA (Lambert *et al*., 1998). The use of these conjugates enables the testing of sera from a wide range of mammalian species, including camelids. ~~A number of commercial ELISA kits are now available and these are being used for large-scale surveys in France and the United Kingdom (Bergonier *et al*., 1997; Nicholas, 1998). In a ring trial of serological tests for *M. agalactiae* organised in 1998 under the auspices of the EC COST Action 826 on ruminant mycoplasmoses, commercial ELISAs performed better than 'home-made' kits.~~

Two commercial ELISA kits for *Ma* are available, one using a fusion protein and the other the whole cells as target antigens. The sensitivities and specificities of the two tests have been calculated as 54% and 100% for the fusion protein and 84% and between 96% in sheep and 99% in goats for the whole cell antigen, respectively (Poumarat *et al.*, 2012). There also appeared to be differences in the ability of the tests to detect the responses to different strains equally. The choice of test depends on the objectives of the proposed study, i.e. a less sensitive test would be sufficient for prevalence study where the disease was endemic while a more sensitive test would be required for disease detection in a disease free region.

ELISAs are not widely available for the other three causative mycoplasmas ~~although 'home-made' assays are carried out by some laboratories~~.

b) Complement fixation

A standard complement fixation test (CFT) for *M. agalactiae* has also been applied to other mycoplasmas involved in the contagious agalactia syndrome (Bergonier *et al*., 1997). Antigens are prepared from washed organisms, standardised by opacity, and lysed, either ultrasonically or by using sodium lauryl sulphate followed by dialysis. Sera are inactivated at 60°C for 1 hour, and the test is carried out in microtitre plates with overnight fixation in the cold or at 37°C for 3 hours. The haemolytic system is added, and the test is read after complete lysis is shown by the antigen control. A positive result is complete fixation at a serum dilution of 1/40 or greater for the following mycoplasmas: *M. agalactiae, Mcc*, and *Mmc.* The CFT is regarded as a herd test and at least ten sera are tested from each herd, preferably from acute and convalescent cases.

Some sera from healthy flocks react in the CFT using M. agalactiae up to a serum dilution of 1/20, but rarely react with the other two antigens. However, in flocks infected with M. agalactiae, sera giving a homologous reaction at 1/80 may cross-react at up to 1/40, the positive threshold, with the other two antigens. It is often difficult to perform the CFT if the quality of the test sera is poor; where possible, the enzyme-linked immunosorbent assay (ELISA) is preferred.

c) Immunoblotting test

~~Immunoblotting tests have been reported as the most sensitive and specific test for *M. mycoides* subsp. mycoides SC, the cause of contagious bovine pleuropneumonia (see Chapter 2.4.9).~~ Immunoblotting tests have also been described for ~~*M. agalactiae*~~ *Ma* and are considered as confirmatory tests for outbreaks in Italy (Nicholas, 1998; Tola *et al*., 1997b). Strong bands at approximately 80 and 55 kDa were seen with sera with antibodies to ~~*M. agalactiae*~~ *Ma*, while sera from healthy flocks show no bands or very faint bands of different sizes. Diluting the sera to 1/50 improves the discrimination between positive and negative sera (Nicholas, 1998). On the other hand, Poumarat *et al.* (2012), using a 1/5 dilution of serum, considered a serum positive for *Ma* from a flock/herd in France if it contained 4 bands: 80, 48, 40 and 30kDa suggesting there may be some geographic differences in humoral responses.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

NB: SECTION C IS ''UNDER STUDY''. THIS IS THE LAST ADOPTED VERSION PUBLISHED IN 2008

Vaccines for the prevention of contagious agalactia due to *M. agalactiae* are used widely in the Mediterranean countries of Europe and in western Asia. No single vaccine has been universally adopted, and no standard methods of preparation and evaluation have been applied.

1. Vaccines for *Mycoplasma agalactiae* infection

a) Inactivated vaccines for *Mycoplasma agalactiae* infection

In Europe, where live vaccines for *M. agalactiae* are not acceptable, attention has focused on the use of killed organisms, mostly using formalin and an adjuvant such as aluminium hydroxide in an oil emulsion. The titres of the preparations, before inactivation, are very high (10^8–10^{10} colony-forming units per ml) and are derived from laboratory strains. Some products are available commercially including a trivalent preparation containing *M. agalactiae, Mcc* and *Mmc* but there are few data on their efficacy. A formalin-inactivated oil emulsion vaccine was shown to be immunogenic and protective in a small trial in lactating sheep and also prevented transmission of *M. agalactiae* (Greco *et al*., 2002).

It is possible that in some instances the apparent lack of protection given by vaccines could be the result of animals being infected with one of the other four mycoplasmas involved in the contagious agalactia syndrome (Gil *et al*., 1999). A multivalent formalin inactivated vaccine incorporating all four causative mycoplasmas and adjuvanted with saponin and aluminium hydroxide appears beneficial in preliminary trials (Ramirez *et al*., 2001).

More recently vaccines inactivated with phenol or with saponin have given superior protection against experimental infections compared with formalin, sodium hypochlorite or heat-inactivated vaccines (Tola *et al*., 1999).

b) Live attenuated vaccines for *Mycoplasma agalactiae* infection

Live attenuated vaccines against *M. agalactiae* have been used in Turkey for many years and have been reported to provide better protection in ewes and their lambs than inactivated vaccines (Nicholas, 2002). However they can produce a transient infection with shedding of mycoplasma. Live vaccines should not be used in lactating animals and should be part of a regional plan in which all flocks from which animals are likely to come into contact be vaccinated at the same time.

2. Vaccines for *Mycoplasma mycoides* subsp. *capri* infection

There is little recent published information on the availability of vaccines for *Mmc* although it is believed that inactivated vaccines are widely used in many Mediterranean countries and in Asia suggesting that their production and use is localised (Bergonier *et al*., 1997). Saponised vaccines have been reported in India which provoke a strong antibody response and show some protection (Sunder *et al*., 2002).

3. *Mycoplasma capricolum* subsp. *capricolum* and *M. putrefaciens*

Although infections with *Mcc* and *M. putrefaciens* can be severe, their prevalence is relatively low and, as might be expected, little or no work appears to have been carried out on preventive vaccination for these infections.

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* *

NB: There is an OIE Reference Laboratory for contagious agalactia
(see Table in Part 4 of this *Terrestrial Manual* or consult the OIE web site for the most up-to-date list: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/ http://www.oie.int/).
Please contact the OIE Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for contagious agalactia

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 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 the Arabian Peninsula, throughout most of the Near East and Middle East, and in Central and South-East Asia.*

The clinical disease resembles rinderpest in cattle. It is usually acute and characterised by pyrexia, serous ocular and nasal discharges, *diarrhoea and pneumonia, and erosive lesions on different mucous membranes particularly in the mouth. At necropsy, erosions may be noted in the gastrointestinal and urogenital tracts. The lungs may show interstitial bronchopneumonia and often secondary bacterial pneumonia. PPR can also occur in subclinical form.*

PPR must be confirmed by laboratory methods, ~~differentiated from~~ as rinderpest, bluetongue, foot and mouth disease and other ~~exanthemous~~ erosive or vesicular conditions as well as contagious caprine pleuropneumonia, can cause clinically similar disease.

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 recommended specimens from live animals are swabs of conjunctival discharges, nasal secretions, buccal and rectal mucosae, and anticoagulant-treated 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: *~~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~~ Effective live attenuated PPR virus vaccines ~~which is~~ are now widely ~~commercially~~ available. Since the global eradication of rinderpest, heterologous vaccines should not be used to protect against PPR.*

A. INTRODUCTION

Peste des petits ruminants (PPR) is an acute viral disease of small ruminants characterised by fever, oculo-nasal discharges, stomatitis, diarrhoea and pneumonia with foul offensive breath. 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 immunosuppression that is induced by the PPR virus (PPRV). PPRV is transmitted mainly by aerosols between animals living in close contact (Lefevre & Diallo, 1990). Infected animals present clinical signs similar to those historically seen with rinderpest in cattle, although the two diseases are caused by distinct virus species.

On the basis of its similarities to rinderpest, canine distemper and measles viruses, 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. The PPR genome also encodes two nonstructural proteins, C and V.

PPR was first described in Côte d'Ivoire (Gargadennec & Lalanne, 1942), but it occurs in most African countries from North Africa to Tanzania (Kwiatek *et al.*, 2011; Lefèvre & Diallo, 1990; Swai, *et al.*, 2009), and in nearly all Middle Eastern countries to Turkey. (Furley *et al.*, 1987; Lefèvre *et al.*, 1991; Ozkul *et al.*, 2002; Perl *et al.*, 1994; Taylor *et al.*, 1990). PPR and is also widespread in countries from a central Asia to South and South-East Asia (reviewed in Banyard et *al.,* 2010; Shaila *et al.*, 1989; Wang *et al.*, 2009).

The natural disease affects mainly goats and sheep. It is generally admitted considered that cattle can are only be naturally infected subclinically. However, in poor conditions it might be possible for cattle to develop lesions following PPRV infection, clinical signs of which would be ascribed to rinderpest. Indeed, although in the 1950s, disease and death were recorded in calves experimentally infected with PPRV-infected tissue and (Mornet *et al.*, 1956). In addition, the virus that was involved in what was thought to be a natural rinderpest outbreak in cattle, sheep and goats in 1971 in Sudan was later shown to be 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). Antibodies to PPRV as well as PPRV antigen and nucleic acid were detected in some samples from an epizootic disease that affected one humped camels dromedaries in Ethiopia and Sudan (Abraham *et al.*, 2005; Ismail *et al.*, 1992; Khalafalla et *al.* 2010; Kwiatek *et al.*, 2011; Roger *et al.*, 2000; 2001). Cases of clinical disease have been reported in wildlife resulting in deaths of wild small ruminants (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 with PPRV (Hamdy & Dardiri, 1976). Dual infections can occur with other viruses such as pestivirus or goatpox virus.

The incubation period is typically 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. 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 very high case fatality in severe cases. However, morbidity and mortality may be much lower in milder outbreaks, and the disease may be overlooked. A tentative diagnosis of PPR can be made on clinical signs, but this diagnosis is considered provisional until laboratory confirmation is made 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 that 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 red areas of congestion or haemorrhage may occur along the longitudinal mucosal folds of the large intestine and rectum (zebra stripes), but they are not a consistent finding. Erosive or haemorrhagic enteritis is usually present and the ileo-caecal junction is commonly involved. Peyer's patches may be necrotic. Lymph nodes are enlarged, and the spleen and liver may show necrotic lesions.

There are no known health risks to humans working with PPRV as no report of human infection with the virus exists. Laboratory manipulations should be carried out at an appropriate containment level determined by biorisk analysis (see Chapter 1.1.3 *Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities*).

B. DIAGNOSTIC TECHNIQUES

A list including all the types of tests available for peste des petits ruminants is given in Table 1. Assays applied to individuals or populations may have different purposes, such as confirming diagnosis of clinical cases, determining infection status for trade and/or movement, estimates of infection or exposure prevalence (surveillance) or checking post-vaccination immune status (monitoring).

Table 1. *Test methods available for peste des petits ruminants and their purpose*

Method	Purpose				
	Population freedom from infection	Individual animal freedom from infection	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Competitive ELISA	++	++		+++	+++
Virus neutralisation	+++	+++		+++	+++
RT-PCR			+++		
Real-time RT-PCR (QRT-PCR)			+++		
Virus isolation in cell culture			++		
Immunocapture ELISA			+++		
Agar gel immunodiffusion			±		±
Counter immunoelectrophoresis			±		

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose.
Although not all of the tests listed as category +++ or ++ have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.
ELISA = enzyme-linked immunosorbent assay; RT-PCR = reverse transcription polymerase chain reaction.

1. Collection of samples

Samples for virus isolation must be kept chilled in transit to the laboratory. 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 (either ethylene diamine tetra-acetic acid or heparin can be used as anticoagulant, though the former is preferred for samples that will be tested using PCR). At necropsy, samples from two to three animals should be collected aseptically from lymph nodes, especially the mesenteric and bronchial nodes, lungs, spleen and intestinal mucosae, chilled on ice and transported under refrigeration. Samples of organs collected for histopathology are placed in 10% neutral buffered formalin. It is good practice to collect blood for serological diagnosis at all stages, but particularly later in the outbreak.

2. Identification of the agent

a) 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 infected mesenteric or bronchial lymph nodes, spleen or lung material and ground up as 1/3 suspensions (w/v) in buffered saline (Durojaiye *et al.*, 1983). 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).

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) Six 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 at room temperature (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.

b) 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.

c) Immunocapture enzyme-linked immunosorbent assay

Advice on the use and applicability of the immunocapture enzyme-linked immunosorbent assay (icELISA) method is available from the OIE Reference Laboratories for PPR. ~~Both~~ The method~~s~~ described is ~~are~~ available as a commercial kit~~s~~.

The icELISA (Libeau *et al.*, 1994) using two monoclonal antibodies (MAb) raised to the N protein allows a rapid identification of PPRV. The instructions provided by kit supplier should be followed, but the following shows a typical procedure for the test.

i) Microtitre ELISA plates ~~(such as Nunc Maxisorp)~~ are coated with 100 µl of a capture MAb solution (diluted according to the instructions of the kit supplier). Coating may be overnight at 4°C or for 1 hour at 37°C.

ii) After washing, 50 µl of the sample suspension is added to each of two wells, and two block (control) wells are filled with buffer.

iii) Immediately add 25 µl of a detection biotinylated MAb for PPR and 25 µl of streptavidin/peroxidase to two 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 0.03% (v/v) hydrogen peroxide is added, and the plates are incubated for 10 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 blank control as three times the mean absorbance values of the control wells.

The test is very specific and sensitive (it can detect $10^{0.6}$ $TCID_{50}$/well of PPRV). The results are obtained in 2 hours.

A sandwich form of the immunocapture ELISA is widely used in India (Singh *et al.*, 2004~~a~~): 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)~~.

d) Nucleic acid recognition methods

Reverse transcription PCR (RT-PCR) techniques based on the amplification of parts of the N and F protein genes have been developed for the specific diagnosis of PPR (Couacy-Hymann *et al.*, 2002; Forsyth & Barrett, 1995). This technique is 1000 times more sensitive than classical virus titration on Vero cells (Couacy-Hymann *et al.,* 2002) with the advantage that results are obtained in 5 hours, including the RNA extraction, instead of 10–12 days for virus isolation. The two most commonly used protocols are given in some detail below. 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 (Saravanan *et al.,* 2004~~; 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 RT-PCR-ELISA is ten times more sensitive than the classical RT-PCR. In recent years, nucleic acid amplification methods for PPR diagnosis have been significantly improved with quantitative real-time RT-PCR (~~Adombi *et al.*, 2011; Balamurugan *et al.*, 2010;~~ e.g. Bao *et al.,* 2008; Batten *et al.,* 2011; Kwiatek *et al.,* 2010). This method is also ten times more sensitive than the conventional RT-PCR, as well as minimising the risk of contamination. The application of nucleic acid isothermal amplification to PPR diagnosis has also been described (Li *et al.*, 2010). The sensitivity of this assay seems to be similar to that of the real-time RT-PCR. This assay is simple to implement, rapid and the result can be read by naked eye.

Because this is a rapidly developing field, users are advised to contact the OIE and FAO[1] Reference Laboratories for PPR (see Table given in Part 4 of this *Terrestrial Manual*) for advice on the most appropriate techniques.

- **RT-PCR for the diagnosis of PPRV based on the amplification of part of the N gene**

N gene amplification is based on the initial protocol described by Couacy-Hymann *et al.* (2002) in a one-step RT-PCR method available as a commercial kit. The test described requires the following materials: Qiagen One-step RT-PCR kit, distilled water and primers.

i) Sequence of primer used:

Primer	**Sequence**
NP3:	5'-GTC-TCG-GAA-ATC-GCC-TCA-CAG-ACT-3':
NP4:	5'-CCT-CCT-CCT-GGT-CCT-CCA-GAA-TCT-3').

ii) Prepare each primer dilution by adding 5 µl of the primer stock solution (100 µM) to 45 µl of distilled water. A primer concentration of 10µM is obtained with a final volume of 50 µl.

iii) Add 5 µl of RNA template to 45 µl of PCR master mix containing:

Reagent	**Mix (1 reaction)**	**Final concentration**
Distilled water	15 µl	
5× RT-PCR Buffer	10 µl	1×
dNTP Mix Qiagen	2 µl	
Q solution	10 µl	
Primer NP3 (10 µM)	3 µl	0.6 µM
Primer NP4 (10 µM)	3 µl	0.6 µM
Qiagen Enzyme mix	2 µl	
Final volume	50 µl	

iv) Distilled water (5 µl) is used in place of RNA to provide a negative control which has to be included into each set of PCR tests.

v) Thermal cycler conditions are set as follows:

50°C for 30 minutes	1 cycle	reverse transcription step

1 Food and Agriculture Organization of the United Nations.

95°C for 15 minutes	1 cycle	Inactivates RT and activates polymerase
94°C for 30 seconds 60°C for 30 seconds 72°C for 1 minute	40 cycles	PCR amplification of the cDNA
72°C for 5 minutes	1 cycle	Final extension
4°C (indefinite)	–	–

vi) The RT-PCR gives an amplification product of 351 bp. 10 µl of these products are analysed by electrophoresis on 1.5 % agarose gel. For all positive results, 40 µl of the final product may be directly used for sequencing.

- **RT-PCR for the diagnosis of PPRV based on the amplification of part of the F gene**

This assay is based on that originally published in Forsyth & Barrett (1995).

i) Sequences of primers used in this protocol:

Primer	**Sequence**
F1b	5'-AGT-ACA-AAA-GAT-TGC-TGA-TCA-CAG-T-3'
F2d	5'-GGG-TCT-CGA-AGG-CTA-GGC-CCG-AAT-A-3'
F1	5'-ATC-ACA-GTG-TTA-AAG-CCT-GTA-GAG-G-3'
F2	5'-GAG-ACT-GAG-TTT-GTG-ACC-TAC-AAG-C-3'

ii) The first stage of this assay uses the Superscript III One-Step RT-PCR Platinum Taq HiFi kit (Life Technologies) and amplification is achieved by using PPRV primers F1b and F2d designed against the PPRV F gene. The following is required per reaction:

2× reaction mix	12.5 µl
Enzyme mix	0.5 µl
RNAse free distilled water	5 µl
Primer F1b (10µM)	1 µl
Primer F2d (10µM)	1 µl
Total Volume	20 µl

Note 1: all reagents except primers and RNAse free distilled water are supplied in the Superscript III One-Step RT-PCR Platinum Taq HiFi kit

Note 2: A mastermix for the required number of reactions can be prepared.

iii) Combine 20 µl reaction mix with 5 µl RNA in a 0.5 ml PCR tube. Each assay requires, at a minimum, the sample, negative control, positive control and no-template control (RNase-free water instead of RNA sample).

iv) Transfer the reactions to a thermal cycler and start the following programme:

50°C for 30 minutes	1 cycle	Reverse Transcription step
94°C for 2 minutes	1 cycle	Inactivates RT and activates polymerase
94°C for 1 minute 55 °C for 1 minute 72°C for 1 minute	35 cycles	PCR amplification of the cDNA
72°C for 7 minutes	1 cvcle	Final extension
4°C (indefinite)	–	–

v) Analyse 10 µl of the reaction product by agarose gel electrophoresis using a 2% agarose gel in either TBE or TAE buffers. If present, PPRV RNA will be amplified to give a DNA fragment of 447 bp. If no DNA product, or a very weak DNA product, is seen, a second round of PCR can be completed to increase the amount of PCR product. This is carried out using the Taq PCR Master Mix (Life Technologies) and primers F1 and F2. The following is required per reaction:

Taq Mastermix	12.5 µl
Nuclease free distilled water	9.5 µl
Primer F1 (10µM)	1 µl

Primer F2 (10µM)	1 µl
Total Volume	24 µl

Note: A mastermix for the required number of reactions can be prepared.

vi) Combine 24 µl reaction mix with 1 µl of the first stage PCR in a 0.5 ml PCR tube. Each assay requires, at a minimum, the sample, negative control, positive control and no-template control (RNase-free water instead of PCR product). Transfer the reactions to a thermal cycler and start the following programme:

94°C for 3 minutes	1 cycle	Activates polymerase
94°C for 1 minute 55°C for 1 minute 72°C for 1 minute	35 cycles	PCR amplification of the cDNA
72°C for 10 minutes	1 cycle	Final extension
4°C (indefinite)	–	–

vii) Analyse 10 µl of the reaction product by agarose gel electrophoresis using a 2% agarose gel in either TBE or TAE buffers. If present, PPRV RNA will be amplified to give a DNA fragment of 371 bp.

viii) DNA product remaining from positive samples identified in (e) or (g) can be purified and subjected to DNA sequencing.

e) 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; Lefèvre & Diallo, 1990)~~.

PPRV may be isolated in primary lamb kidney/lung cells and some cell lines (Vero, B95a). Unfortunately, PPRV isolation using such cells is not always successful on first passage and may require multiple blind passages. Recently derivatives of cell lines (Vero, CV1) expressing the morbillivirus receptor, the signalling lymphocyte activation molecule (SLAM or CD150), have been developed that can enable isolation of field viruses from pathological specimens in less than 1 week, without requirement for blind passages. These include a derivative of the monkey cell line CV1 expressing goat SLAM (Adombi *et al.*, 2011) and derivatives of Vero cells expressing dog SLAM. 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 cell lines expressing SLAM. In unmodified Vero cells, it is sometimes difficult to see the syncytia. If they exist, they are very small. However, small syncytia are always seen in infected Vero cells stained with haematoxylin and eosin. Syncytia are recognised by a circular arrangement of nuclei giving a 'clock face' appearance. Cover-slip cultures may show CPE earlier than day 5. Some cells may contain intracytoplasmic and intranuclear inclusions, others may be 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.

3. Serological tests

The demonstration of antibodies in PPRV infected goats and sheep can be used to support a diagnosis ~~by the antigen antibody detection~~ based on clinical signs, but such antibodies may also arise from vaccination with any of the current PPRV vaccines. 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 now usually carried out in 96-well microtitre plates ~~(Rossiter *et al.*, 1985)~~ although roller-tube cultures may also be used. Vero cells are preferred, but primary lamb kidney cells may also be used.

This test requires the following materials: 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.

i) Dilute the sera ~~at~~ 1/5, and then make twofold serial dilutions in cell culture medium.

ii) 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.

iii) 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 medium per well. Make the wells containing the virus dilutions up to 200 µl with complete culture medium, and incubate the plates for 1 hour at 37°C.

v) Add 50 µl of cell suspension to each well, pat the sides of the plate lightly to distribute the cells in the well and cover. Incubate the plates at 37°C in the presence of CO_2.

vi) Read the plates after 1 and 2 weeks of incubation. The results should be as follows:

If the virus dilution has been done correctly, all the virus control wells with 100 and 10 $TCID_{50}$/well will show CPE, 50% of the wells will show CPE for the 1 $TCID_{50}$/well dilution, and none should show CPE for the 0.1 $TCID_{50}$/well dilution. The test is only valid if the virus has been suitably diluted.

For the serum titration, there will be no CPE in wells where the virus had been neutralised by serum during the test; any level of CPE means that the virus had not been neutralised by serum. The neutralising titre is the dilution of serum that neutralises virus in half the wells. A neutralising titre of greater than 10 is positive.

b) Competitive enzyme-linked immunosorbent assay

Several competitive ELISAs (C-ELISA) have been described, based on the use of MAbs that recognise virus proteins. They are of two types: those where the MAb recognises the N protein and use recombinant N protein produced in baculovirus as the antigen (~~Choi *et al.*, 2005;~~ e.g. Libeau *et al.*, 1995); and those with a viral attachment protein (H) specific MAb and antigen consisting of purified or part-purified PPRV (vaccine strain) (e.g. Anderson & McKay, 1994; Saliki *et al.,* 1993~~; Singh *et al.*, 2004b~~). All the assays work on the principle that antibodies to PPRV in test sera can block the binding of the MAb to the antigen.

Advice on the use and applicability of ELISA methods is available from the OIE Reference Laboratories for PPR. Some methods are available as commercial kits; these are the only practicable way to carry out this test. Before use laboratories should seek assurance that the kit has been validated in accordance with the OIE Validation Standard (see chapter 1.1.5). The only alternative would be for a laboratory to develop and validate all the reagents (monoclonals and antigens) in house. ~~An example protocol for one method (Libeau *et al.*, 1995) is given below.~~

~~i) Coat microtitre plates (such as Nunc 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 + 0.05% Tween 20 + 0.5% fetal calf serum) to all wells, and then add 5 µl of test sera to test wells in duplicate (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 to working strength as advised by the supplier, 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 = 1 – (absorbance of the test wells/absorbance of the MAb control wells) × 100~~

~~Sera showing PI greater than 50% in both duplicate wells are positive.~~

C. REQUIREMENTS FOR VACCINES

1. Background

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 *Principles of veterinary vaccine production*. The guidelines given below and in chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements.

a) Rationale and intended use of the product

Sheep and goats vaccinated with an attenuated strain of PPR or that recover from PPR develop an active life-long immunity against the disease (Durojaiye, 1982). Several homologous PPR vaccines are available, being cell culture-attenuated strains of natural PPRV (Sen *et al.,* 2010). In 1998, the OIE World Assembly (formally OIE International Committee) endorsed the use of such a 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 also been three published reports on the preliminary results from recombinant capripox-based PPR vaccines that are able to protect against both capripox and PPR (Berhe *et al.*, 2003; Chen *et al.,* 2010; Diallo *et al.*, 2002). The production and validation of vaccine from the commercially available attenuated PPRV is described here.

2. Outline of production and minimum requirements for conventional vaccines

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.~~

i) *Biological characteristics*

The history of the vaccine received and stored in the laboratory as master seed should be well known and registered: origin, level of passages in cell culture, the range of the number of passages of the vaccine in cell culture that has been tested and shown to be effective in providing protection in animals against PPR with the recommended dose for vaccination for at least 3 years. This PPR virus vaccine strain should not be able to be excreted by inoculated animals and spread to in-contact animals. It should be proven that the vaccine strain has not reverted to virulence following at least three back passages in sheep and goats (Diallo *et al.,* 1989).

ii) *Quality criteria* (*sterility, purity, freedom from extraneous agents*)

The seed should be controlled and tested free from bacterial, fungus and mycoplasma contamination. It should be tested free from pestivirus and any other known extraneous virus. Only live attenuated PPR virus should be present. The seed should have passed an innocuity test in animals (rodents, sheep and goats) and have demonstrated its efficacy to protect sheep and goats against PPR with the recommended dose.

b) Method of ~~culture~~ manufacture

i) *Procedure*

Once the manufacturer has received samples from an institution holding the PPRV vaccine bank – the master seed – it must prepare primary and secondary working seed batches. The production seed batch, from which the final vaccine is produced, is prepared from the secondary working seed. By preparing primary and secondary working seeds, the need to carry out a large number of passages after the master seed is avoided for the vaccine production. In this way, it is possible to comply with one of the OIE recommendations: 5–10 passages maximum after the master seed (see chapter 1.1.6).

~~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~~

a) *Primary and secondary working seed batches:* ~~This consists of virus in its 70th passage in Vero cells (PPRV 75/1 LK6 BK2 Vero 70).~~ When preparing primary and secondary working seed batches it is important to avoid infecting the cells with high doses of virus (high multiplicity of infection [m.o.i.]), as this will lead to the accumulation of defective particles in the viral suspension produced, which will diminish the titre of subsequent products. On the other hand, very weak m.o.i. (e.g. 0.0001) will prolong the culture time.

The freeze-dried contents of a flask from the master seed are reconstituted with 2 ml of ~~sterile water (or~~ cell culture medium without serum~~)~~. This liquid is mixed with Vero cells, if the vaccine is produced on Vero cells, suspended in complete culture medium to provide at least 0.001 $TCID_{50}$ per cell. Cell culture ~~dishes~~ flasks are filled with this virus/cell mixture (around 2×10^7 Vero cells in a 175 cm^2 ~~dish~~ flask), 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 carried out every 2 days until the CPE reaches 70–80%, which is the time for final freezing of the culture ~~dishes~~ flasks (in general, at least two further harvestings can be made before final freezing of the culture flasks). All suspensions of virus collected are submitted to two freeze–thaw cycles, then added to form a single batch, which serves as the working 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.

~~o **Preparation of the working seed batch**~~

b) *Preparation of the production seed batch:* ~~This is done~~ The production seed batch is prepared under the same conditions as for the ~~primary seed batch~~ working seed batch apart from the fact that infection of the cells can be done with a higher m.o.i. compared with the previous cases and usually with at least m.o.i. 1–10 ~~CPE~~. 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 7–10 days ~~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 4 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.

d) *Vaccine production:* This operation is performed on a larger scale. Cells can be infected with virus at a m.o.i. 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.

e) *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 innocuity, 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.

~~Instead of the Weybridge medium, it has been demonstrated that dehydration of an attenuated PPR vaccine in an excipient containing trehalose dramatically improves the thermostability of the final that can resist at 45°C for many days with minimal loss of potency (Sakar *et al.*, 2003; Silva *et al.*, 2011; Worrall *et al.*, 2001).~~

ii) *Requirements for substrates and media*

- *Cells:* Cells used for the production of PPR vaccine must be free from bacterial, fungal and viral contaminations. The source of these cells should be known and documented.

- *Serum:* The serum used in the cell culture should free from adventive virus, in particular pestiviruses. It is recommended to use irradiated serum. The country origin of the serum should be known (it should be avoided to use the serum from countries with high risk of transmissible spongiform encephalopathies (TSE) infections.

- *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.

iii) *In-process controls*

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 CO2. The plates are read (by examining for CPE) 7–10 ~~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~~

iv) *Final product batch tests*

Sterility and purity

~~a) Identity~~

Tests for sterility and freedom from contamination of biological materials may be found in chapter 1.1.7.

The content 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~~ It should be tested and proven to be non-contaminated by bacteria, fungus or other viruses. The test carried out 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 1.1.7.

Safety and efficacy

A safety test should be done in rodents in order to detect any nonspecific toxicity associated with the product and also on host recipient of the vaccine, currently sheep and goats. ~~The test requires reconstituted vaccine in solvent (mixed contents of five bottles).~~

For rodents, six guinea-pigs, each weighing 200–250 g; ten unweaned mice (17–22 g, Swiss line or similar). The vaccine contents of five bottles are mixed and used. The 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.

Normally the minimum immunising dose is 100× the lowest dose of vaccine virus able to induce a 50% immunising response. For example, in the case of the attenuated Nigeria 75/1 vaccine the required minimum titre per dose has been shown to be $10^{2.5}$ $TCID_{50}$.

~~**d) Potency and efficacy in small ruminants**~~

~~This~~ For the target host safety test, ~~requires~~ the ~~following:~~ PPR vaccine must be reconstituted with normal saline. The mixed contents of five bottles are reconstituted in the diluent ~~in a way~~ to give solutions of ~~provide~~ 100 doses/ml ~~and~~ and 0.1 dose/ml. Six goats and six sheep are used, all approximately 1-year old and free from ~~antibodies to rinderpest or~~ PPR antibodies~~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~~ subjected to daily clinical examination for 3 weeks including recording of rectal temperatures. 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 previously tested for inducing PPR clinical signs in sheep and goats ~~(10^3 LD50 per animal)~~. The animals are observed and their body temperature measurements are recorded daily for 2 weeks.

The vaccine is considered as safe if no abnormal clinical signs are observed in the vaccinated animals, in particular those which have received the highest dose. ~~and if in-contact animals have remained susceptible to the challenge virus.~~

Its efficacy is proven if all vaccinated animals resist the challenge while the in-contact animals remain susceptible to the challenge virus. ~~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~~

Batch potency

The potency of each batch should be determined by virus titration in cell culture and shown to meet the minimum immunising dose requirements in the efficacy test.

The potency of the vaccine is proven if animals vaccinated with the dose used in the efficacy test (see above) develop antibody against PPRV when tested 3 weeks after vaccination by virus neutralisation at a serum dilution of > 1/10). The titration of neutralising antibody is carried out as described in section B.3.a above.

~~**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.~~

It might possible that no seroconversion is detected in some animals 3 weeks after vaccination but those animals will resist challenge if the vaccine is efficacious. Resistance in these cases is attributed to the cell-mediated immunity induced by PPRV (Saravanan *et al.*, 2010).

c) Requirements for authorisation

i) *Safety requirements*

Target and non-target animal safety

The vaccine should be safe for use in all species ~~breeds~~ of targeted animals, including young and pregnant recipients.

Reversion-to-virulence for attenuated/live vaccines

Information should be available to indicate that studies have been carried out and have demonstrated that the vaccine strain which has been used has not reverted to virulence after at least three back passages.

Environmental consideration

PPR vaccine should not be excreted by the inoculated animals.

ii) *Efficacy requirements*

For animal production

Field or other tests should have proven that the attenuated PPR vaccine is safe for use in all sheep and goats species, including young and pregnant animals.

For control and eradication

Sheep and goats which have recovered from a PPR infection appear to be protected against a subsequent infection for the rest of their lives. Neutralising antibodies anti PPRV were found in sheep and goats up to three years after vaccination with the attenuated PPRV vaccine strain Nigeria 75/1. Other attenuated PPR vaccines that have been developed in India have been shown to produce strong immunity too (Saravanan *et al.*, 2010; Sen et *al.*, 2010). All these information indicate that PPR is a disease that can be well controlled, even eradicated following mass and well planned vaccination campaign as that was done for rinderpest. There is an example of a country which has succeeded in the implementation of this strategy.

iii) *Stability*

PPR vaccine freeze-dried in the Weybridge medium 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.

iv) *Vaccines permitting a DIVA strategy (detection of infection in vaccinated animals)*

No DIVA vaccine and associated test is currently available for field use.

v) *Duration of immunity*

Duration of immunity should be determined for each vaccine strain in animal trials. For the Nigeria 75/1 vaccine, this has been shown to be at least 3 years.

~~• **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.~~

3. Vaccines based on biotechnology

a) Vaccines available and their advantages

Preliminary results on recombinant capripox-based PPR vaccines indicate that they can protect against both capripox and PPR (Berhe *et al.*, 2003; Diallo *et al.*, 2002, Chen *et al.,* 2010). They are not yet validated for field use.

b) Special requirements for biotechnological vaccines, if any

None.

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NB: There are OIE Reference Laboratories for Peste des petits ruminants
(see Table in Part 4 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list:
http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/ http://www.oie.int/).
Please contact the OIE Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for Peste des petits ruminants

CHAPTER 2.8.9.

SWINE VESICULAR DISEASE

SUMMARY

Swine vesicular disease (*SVD*) *is a contagious disease of pigs, caused by an enterovirus and characterised by vesicles on the coronary bands, heels of the feet and occasionally on the lips, tongue, snout and teats. Strains of SVD virus may vary in virulence, and the disease may be subclinical, mild or severe, the latter usually only being seen when pigs are housed on abrasive floors in damp conditions. The main importance of SVD is that it is clinically indistinguishable from foot and mouth disease* (*FMD*), *and any outbreaks of vesicular disease in pigs must be assumed to be FMD until investigated by laboratory tests and proven otherwise. However, subclinical infection has been the most frequent condition observed during recent years.*

Identification of the agent: *Where a vesicular condition is seen in pigs, the demonstration by enzyme-linked immunosorbent assay* (*ELISA*) *of SVD viral antigen in a sample of lesion material or vesicular fluid is sufficient for a positive diagnosis. If the quantity of lesion material submitted is not sufficient* (*less than 0.5 g*), *or if the test results are negative or inconclusive, a more sensitive test, such as the reverse transcription polymerase chain reaction* (*RT-PCR*) *or isolation of virus* (*VI*) *in porcine cell cultures, may be used. If any inoculated cultures subsequently develop a cytopathic effect, the demonstration of SVD viral antigen by ELISA or viral RNA by RT-PCR will suffice to make a positive diagnosis. Subclinical infection may be detected by random sampling of pen-floor faeces followed by identification of SVD viral genome using RT-PCR or VI tests.*

Serological tests: *Serological tests can be used to help confirm clinical cases as well as to identify subclinical infections. Specific antibody to SVD virus can be identified using the microneutralisation test or ELISA. Although the microneutralisation test requires 2–3 days to complete, it remains the definitive test for antibody detection ~~to SVD virus~~. A small proportion* (*up to 0.1%*) *of normal, uninfected pigs will react positively in serological tests for SVD. The reactivity of these singleton reactors is transient, so that they can be differentiated from infected pigs by resampling of the positive animal and its cohorts.*

Requirements for vaccines ~~and diagnostic biologicals~~: *There are currently no commercial vaccines available against SVD. Diagnostic and standard reagents are available from ~~regional~~ reference laboratories.*

A. INTRODUCTION

Swine vesicular disease (SVD) can be a subclinical, mild or severe vesicular condition depending on the strain of virus involved, the route and dose of infection, and the husbandry conditions under which the pigs are kept. Clinically, SVD is indistinguishable from foot and mouth disease (FMD) and this is its main importance. It is therefore urgent that cases of SVD be distinguished from FMD by laboratory investigation. Recent outbreaks of SVD have been characterised by less severe or no clinical signs; infection has been detected when samples are tested for a serosurveillance programme or for export certification.

The incubation period for SVD is between 2 and 7 days, after which a transient fever of up to 41°C may occur. Vesicles then develop on the coronary band, typically at the junction with the heel. These may affect the whole coronary band resulting in loss of the hoof. More rarely, vesicles may also appear on the snout, particularly on the dorsal surface, on the lips, tongue and teats, and shallow erosions may be seen on the knees. Affected pigs may be lame and off their feed for a few days. Abortion is not a typical feature of SVD. Recovery is usually complete in 2–3 weeks, with the only evidence of infection being a dark, horizontal line on the hoof where growth has been temporarily interrupted. The clinical signs vary according to the age of pigs affected, the conditions under which they are kept and the strain of SVD virus involved (Loxam & Hedger, 1983). Disease caused by mild strains may remain unobserved, particularly in pigs kept on grass or housed on deep straw. Younger animals are more

severely affected, although mortality due to SVD is very rare, in contrast with FMD in young stock. Nervous signs have been reported, but are unusual. Affected pigs may excrete virus from the nose and mouth and in the faeces up to 48 hours before the onset of clinical signs. Most virus is produced in the first 7 days after infection, and virus excretion from the nose and mouth normally stops within 2 weeks. Virus may continue to be shed for up to 3 months in the faeces. The SVD virus is extremely resistant to inactivation in the environment, and is stable in the pH range 2.5–12.0 (Mann, 1981). This is in contrast to the FMD virus, which is very labile outside the pH range 6.0–8.0.

Because SVD may be mild or subclinical, it is essential when submitting samples from suspect clinical cases that serum samples from both the suspect pigs and other apparently unaffected animals in the group be included. It is possible for SVD to circulate unnoticed until it affects a particularly susceptible group, and therefore, in order to ascertain how long infection has been present, it is necessary to look for seroconversion to SVD virus in apparently healthy animals. Also the identification of the isotype of the immunoglobulins (M or G) to SVD virus may help to ascertain the time of exposure to infection.

SVD is clinically very similar to FMD. Samples for virus isolation or antigen detection must be handled and submitted as though they contained FMD virus and must be transported in 0.04 M phosphate buffered saline (PBS) mixed with glycerol (1/1), pH 7.2–7.6, with antibiotics such as (final concentration per ml) penicillin (1000 International Units [IU]), neomycin sulphate (100 IU), polymyxin B sulphate (50 IU), and mycostatin (100 IU) (Kitching & Donaldson, 1987).

SVD virus (SVDV) has been classified as a pig enterovirus, in the family *Picornaviridae*. All isolates are classified in a single serotype, with four distinguishable antigenic/genomic variants (Brocchi *et al*., 1997), which evolved sequentially in different time-periods without overlapping, except for the third and fourth variants that were co-circulating in Italy during 1992–1993. All SVD viruses occurring since then diverge from a common origin and cluster in a unique antigenic/genomic lineage corresponding to the fourth and most recent group; however, two genomic sub-lineages are distinguishable within it (Knowles *et al*., 2007). Antigenically, SVD virus is related to the human virus coxsackievirus B5. There are reports of seroconversion to SVD virus in laboratory workers handling the agent. Clinical disease was reported to be mild with the exception of a single case of meningitis associated with SVD virus infection. However, there have been no reported cases of seroconversion or disease in farmers or veterinarians working with infected pigs. Under experimental conditions, it has not been possible to show transmission of coxsackievirus B5 between pigs. Laboratory manipulations should be carried out at an appropriate biosafety and containment level determined by biorisk analysis (see Chapter 1.1.3 *Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities*).

B. DIAGNOSTIC TECHNIQUES

Table 1. *Test methods available for swine vesicular disease and their purpose*

Method	Purpose						
	Population freedom from infection and re-establishment of freedom after outbreaks	Individual animal freedom from infection	Serological confirmatory test	Confirmation of clinical cases	Detection of subclinical infection	Prevalence of infection – surveillance	Immune status in individual animals
Virus isolation	–	+	–	+++	+++	–	–
RT-PCR	–	+++	–	+++	+++	–	–
ELISA for antigen detection	–	–	–	++	–	–	–
Virus neutralisation	–	–	+++	+	+	–	+++
Competitive ELISA for Ab screening	+++	–	–	+	+	+++	+++
ELISA for IgG and IgM identification	+	–	–	+	+	–	+++

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose.
Although not all of the tests listed as category +++ or ++ have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.
RT-PCR = reverse-transcription polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay.

1. Identification of the agent

Any vesicular condition in pigs may be FMD. Once suspicion of FMD has been eliminated, the diagnosis of SVD requires the facilities of a specialised laboratory. Countries that lack such a facility should send samples for investigation to an OIE Reference Laboratory for SVD[1] (see Table in Part 4 of this *Terrestrial Manual* or consult the OIE web site for the most up-to-date list: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/). In the Americas, parallel testing for vesicular stomatitis viral antigen should also be conducted.

The detection of antigens or genome of SVD virus by means of enzyme-linked immunosorbent assay (ELISA) and reverse transcription polymerase chain reaction (RT-PCR) has the same diagnostic value as virus isolation. Due to their speed, ELISA and RT-PCR make suitable screening tests. However, virus isolation is the reference method and should be used if a positive ELISA or RT-PCR result is not associated with the detection of clinical signs of disease, the detection of seropositive pigs, or a direct epidemiological connection with a confirmed outbreak.

If there are clinical signs, investigation should start with the examination of a 10% suspension of lesion material in phosphate buffered saline (PBS) or tissue culture medium and antibiotics. Faecal samples are the specimen of choice for the detection of virus where subclinical SVD is suspected. Faecal samples can be collected from individual pigs or from the floor of premises suspected to contain, or to have contained, pigs infected with SVD. The level of virus in faeces is usually insufficient for detection by ELISA and the use of RT-PCR and/or virus isolation is required. A significant proportion of faecal samples inoculated into cell cultures will give rise to the growth of other enteroviruses. These can be differentiated from SVD virus by ELISA or RT-PCR, but they may also outgrow SVD virus that is present, and give rise to false negative results. Therefore, RT-PCR is more sensitive than virus isolation when applied to faecal samples.

o Preparation of samples

Lesion material: a suspension is prepared by grinding the sample in sterile sand in a sterile pestle and mortar with a small volume of PBS or tissue culture medium and antibiotics. Further medium should be added to obtain approximately a 10% suspension. This is clarified by centrifugation at 2000 ***g*** ~~10000 rpm~~ for 20–30 minutes in a high speed centrifuge and the supernatant is harvested.

Faecal samples: faecal material (approximately 20 g) is resuspended in a minimal amount of tissue culture medium or phosphate buffer (0.04 M phosphate buffer or PBS). The suspension is homogenised by vortexing and clarified by centrifugation at 2000 ***g*** ~~10000 rpm~~ for 20–30 minutes in a high speed centrifuge; the supernatant is harvested and filtered through 0.45 µm filter.

a) Virus isolation

A portion of the clarified epithelial or faecal suspension is inoculated on to monolayers of IB-RS-2 cells or other susceptible porcine cells, grown in appropriate containers (25 cm^2 flasks, rolling tubes, 24-, 12-, 6-well plates). For differential diagnosis (e.g. FMD) in case of clinical lesions bovine cell culture systems should also be employed. Generally SVD virus will grow in cells of porcine origin only ~~however there is a report that the virus can be isolated in secondary lamb kidney cells~~. Tissue culture medium is supplemented with 10% bovine serum for cell growth, with 1-3% bovine serum for maintenance, and with antibiotics.

Cultures are examined daily. If a cytopathic effect (CPE) is observed, the supernatant fluid is harvested and virus identification is performed by ELISA (or other appropriate test, e.g. RT-PCR). Negative cultures are blind-passaged after 48 or 72 hours, and observed for a further 2–3 days. If no CPE is evident after the second passage, the sample is recorded “NVD” (no virus detected). When isolating virus from faeces in which the amount of virus present may be low, a third tissue culture passage may be required.

b) Immunological methods

- **Enzyme-linked immunosorbent assay**

~~1 FAO (Food and Agriculture Organization of the United Nations) World Reference Laboratory for FMD, Institute for Animal Health, Pirbright, Woking, Surrey GU24 0NF, United Kingdom (also an OIE Reference Laboratory for FMD and SVD). Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZSLER), Brescia, Via Bianchi 9, Italy.~~

The detection of SVD viral antigen by an indirect sandwich ELISA has replaced the complement fixation test as the method of choice. The test is the same as that used for FMD diagnosis. Wells of ELISA plates are coated with rabbit antiserum to SVD virus. This is the capture serum. Test sample suspensions are added and incubated. Appropriate controls are also included. Guinea-pig anti-SVD detection serum is added at the next stage followed by rabbit anti-guinea-pig serum conjugated to horseradish peroxidase. Extensive washing is carried out between each stage to remove unbound reagents. A positive reaction is indicated if there is a colour reaction on the addition of chromogen (for example orthophenylenediamine) and substrate (H_2O_2). With strong positive reactions this will be evident to the naked eye, but results can also be read spectrophotometrically at the appropriate wavelength, in which case an absorbance reading ≥0.1 above background indicates a positive reaction. As an alternative to guinea-pig and rabbit antisera, suitable monoclonal antibodies (MAbs) can be used, coated to the ELISA plate as the capture antibody, or peroxidase conjugated as ~~tracing~~ detector antibody. For example, a simple sandwich ELISA performed with MAb 5B7 as both catching and conjugated/detector antibody, that represents also the reference method for the serological competitive ELISA, is suited for the detection of SVD viral antigen.

A MAb-based ELISA can also be used to study antigenic variation among strains of SVD virus. Tissue-culture grown viral strains are trapped by a rabbit hyperimmune antiserum to SVD virus adsorbed to the solid phase. Appropriate panels of MAbs are then reacted and the binding of MAbs to field strains is compared with the binding of MAbs to the parental strains. Strong binding indicates the presence of epitopes shared between the parental and the field strains (Brocchi *et al*., 1997).

c) Nucleic acid recognition methods

- **Reverse transcription-polymerase chain reaction**

Reverse transcription followed by the PCR (RT-PCR) is a useful method to detect SVD viral genome in a variety of samples from clinical and subclinical cases. Several methods have been described (Benedetti *et al*., 2010; Blomström *et al*., 2008; Callens & De Clercq, 1999; Fallacara *et al*., 2000; Hakhverdyan *et al*., 2006; Lin *et al*., 1997; McMenamy *et al*., 2011; Nunez *et al*., 1998; Reid *et al*., 2004a; 2004b; Vangrysperre & De Clercq, 1996), employing different techniques for RNA extraction, targeting different parts of the SVD virus genome and using different approaches to detect the DNA products of amplification. The method reported below describes an RNA immune-extraction protocol and a one-step RT-PCR ~~(One step RT-PCR)~~ protocol targeting the SVDV *3D* gene, which codes for the RNA-polymerase.

To isolate RNA the immunocapture technique using a SVD virus-specific MAb has been shown to be particularly effective in the case of faecal samples (Fallacara *et al*., 2000); however, RNA extraction can also be obtained using commercial kits based on chaotropic salt lysis and silica RNA affinity. With the one-step RT-PCR, the reverse transcription and PCR amplification are carried out in the same tube in a single step, minimising the time required and reducing the risk of contamination. A number of commercial one-step RT-PCR kits are available and the kit manufacturer's specific instructions should be followed; an example protocol is given below.

~~In the case of faecal samples, an immunocapture technique using a SVD virus-specific MAb has been shown to be effective (Fallacara *et al*., 2000).~~ This method ~~(described below)~~ is suitable for laboratories without sophisticated equipment for real-time detection of DNA amplification products, but where such facilities are available an approach such as that described by Reid *et al.* (2004a; 2004b) offers advantages in terms of ease of use and reduced risk of laboratory contamination by PCR products. However, in a comparative study on positive faecal samples from many different outbreaks the one step RT-PCR showed the best diagnostic performance, with the capability to reveal all the circulating genomic sub-lineages, with respect to two real-time RT-PCR assays targeting the ~~5'UTR~~ 5'-untranslated region and an RT-loop-mediated Isothermal amplification (LAMP) assay (Benedetti *et al.,* 2010).

- **RNA Immune-extraction**

i) RNA Immune-extraction: Coat wells of an ELISA plate with a saturating solution of MAb 5B7 (200 µl/well, diluted in carbonate-bicarbonate buffer) by overnight incubation at 4°C. Wash plates three times with PBS. Use plates immediately or store at –20°C for up to 2–3 weeks, or more if stabilised. ~~NOTE: As an alternative to immunocapture, RNA extraction can be performed using a suitable commercially available kit (such as RNeasy kit from Qiagen).~~

ii) Distribute each sample (faeces suspension) into three wells of the 5B7-coated plate (200 µl/well, 600 µl of sample in total).

iii) After incubation for 1 hour at 37°C with very slow shaking, wash wells three times with PBS. Washing is performed manually, in order to avoid cross-contamination between wells.

iv) RNA is extracted from each sample by adding approximately 100 µl/well of lysis buffer (4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7, 0.5% Sarkosyl). Incubate wells for 3–5 minutes and recover the sample from the three wells (300–350 µl total), and transfer into a single tube.

v) RNA is then precipitated by adding a mixture of 750 µl of absolute ethanol and 35 µl of 3 M sodium acetate (pH 5.2); vials are vortexed and incubated at –20°C for a minimum of 1 hour (prolonged overnight precipitation at –20°C may also be suitable).

vi) Centrifuge the sample at 15,500–16,000 ***g*** ~~13,000 rpm~~ for 30 minutes at 4°C, after which a pellet should be visible which should be washed with 500 µl of 70% cold ethanol (centrifuged at 13000 rpm for 10 minutes at 4°C) and dried.

vii) Resuspend the RNA pellet in 20 µl of DEPC water, or commercially available RNase-free water.

NOTE: As an alternative to immunocapture, RNA extraction can be performed using a suitable commercially available kit.

~~vii) Assemble the reaction mixes for the reverse transcription of SVDV RNA. Mixes (20 µl total volume) contain 4 µl of 5× AMV reaction buffer (final reaction concentration is 8 mM $MgCl_2$, 30 mM KCl, 1 mM dithiothreithol, 50 mM Tris/HCl, pH 8.5), 2 µl 10 mM dNTP mix, 1 µl Random Primer pd$(N)_6$ (100 pmol), 0.3 µl RNase Inhibitor (12 units), 0.2 µl AMV reverse transcriptase (5 units) 0.5 µl BSA (0.5 µg) and 12 µl of nuclease free water.~~

One step RT-PCR

The following protocol may need to be adjusted to suit particular reagents used.

i) Assemble the reaction mix (20 µl is the final volume for each test sample)

Rnase free water	10.75 µl
5× RT-PCR buffer	5 µl
dNTP mix (10 mM each dNTP)	1 µl
pSVDV-SS4 Forward Primer 10 pmol/ul*	1µl
pSVDV-SA2 Reverse Primer 10 pmol/ul*	1 µl
RNAse inhibitor	0.25 µl (equivalent to 5 U)
RT-PCR enzyme mix	1 µl

*Primers sequence: pSVDV-SS4 5'-TTC-AGA-ATG-ATT-GCA-TAT-GGG-G-3'
pSVDV-SA2 5'-TCA-CGT-TTG-TCC-AGG-TTA-CY-3'

ii) Add 5 µl of each template RNA to 20 µl reaction mix.

iii) Run the following program in a thermal cycler:

One cycle at 50°C for 30 minutes (reverse transcription step)

One cycle at 95°C for 15 minutes (initial activation step)

40 cycles of 94°C for 20 seconds (denaturation), 60°C for 20 seconds (annealing), 72°C for 45 seconds (extension)

One cycle at 72°C for 10 minutes (final extension).

iv) Mix a 20 µl aliquot of each sample with 4 µl of staining solution and load onto a 2% agarose gel. After electrophoresis, a positive result is indicated by the presence of a 154 bp fragment of SVDV RNA polymerase (3D) gene in the gel. Alternatively, gel can be stained after electrophoresis to reduce contamination of equipment by the staining solution.

~~viii) Resuspend the RNA pellet in 20 µl of reverse transcription mix (see above).~~

~~ix) Incubate reactions at 42°C for 60 minutes followed by 95°C for 3 minutes.~~

~~x) PCR for SVDV Genome: Add 5 µl of prepared cDNA to 20 µl PCR amplification mix containing 0.55 µl 2M KCl (final reaction concentration is 44 mM), 2 µl 10 mM dNTP mix, 1 µl pSVDV SA2 rev primer (10 pmol: 5' TCA CGT TTG TCC AGG TTA CC 3'), 1 µl pSVDV SS4 dir primer (10 pmol: 5' TTC AGA ATG ATT GCA TAT GGG G 3'), 0.25 µl Taq polymerase (1.24 units) and 15.2 µl of nuclease free water.~~

~~xi) Place the plate in a PCR thermocycler and run the following programme:~~

~~One cycle at 94°C for 3 minutes,~~

~~40 Cycles of 94°C for 20 seconds, 60°C for 20 seconds, 72°C for 30 seconds,~~

~~One cycle at 72°C for 5 minutes.~~

- **Sequence analyses**

Comparative analysis of sequences of the viral genome is useful to establish relationships between isolates of SVD virus. By sequencing the *1D* gene, which codes for the major structural protein VP1, or the *3D gene* ~~which codes for the RNA-polymerase~~, it has been possible to group strains of SVD virus according to their

sequence homology, and to relate epidemiologically strains causing disease in different regions or at different times (Brocchi *et al*., 1997). The databases of *1D* and *3D* gene sequences of SVD viruses are held at the OIE Reference Laboratory, Pirbright, UK and the OIE Reference Laboratory, Brescia, Italy, respectively. ~~A database of ID gene sequences of SVD viruses is held at the OIE Reference Laboratory, Pirbright, UK.~~

2. Serological tests

These are used in the laboratory confirmation of outbreaks, for serological surveillance and for export certification of pigs. SVD is often diagnosed solely on the evidence of serological tests. Because of the subclinical or mild nature of the disease, it is often first suspected following routine serology for disease surveillance or export certification. The virus neutralisation (VN) test, the double immunodiffusion test, the radial immunodiffusion test, the counter immunoelectrophoresis test and the ELISA have all been described for the detection of antibodies to SVD virus (Brocchi *et al*., 1995; Donaldson *et al*., 1983; Golding *et al*., 1976). However, the VN test and the ELISA are the only techniques commonly used. The VN test is the accepted standard test, but has the disadvantage that it takes 2–3 days to complete and requires tissue culture facilities. The ELISA is more rapid and can be more easily standardised. A small proportion of sera from animals with no previous exposure to SVD virus ~~will~~ may react positively in serological tests for antibody to SVD virus. The 5B7 MAb competitive ELISA (MAC-ELISA) is a reliable technique for detecting SVD antibody (Brocchi *et al*., 1995; Heckert *et al*., 1998) and similar results have been obtained with other ELISAs (Chenard *et al*., 1998; Ko *et al*., 2005). Results from a small proportion, 0.2%-0.4%, of sera from normal pigs are borderline or positive by the MAC-ELISA and should be retested by the VN test. Up to approximately 50% of these sera will also be positive by the VN test (i.e. 0.1–0.2% of the original population). Animals that test positive by ELISA but negative by VN test can be regarded as uninfected. Repeat samples should be collected from animals positive in both tests and from cohorts. A constant or declining titre in the positive animal and the absence of antibody to SVD virus in cohorts confirms the status of the positive animal as a 'singleton reactor'. The factors responsible for 'singleton reactors' are unknown. Serological cross-reactivity with SVD virus might arise due to infection with another, as yet unidentified, picornavirus or may be due to other non-specific factors present in the serum. Identification of the isotype of antibody present in positive sera (Brocchi *et al*., 1995) can be helpful ~~as sera from infected pigs usually contain specific IgG alone or both IgG and IgM, where~~ as sera from 'singleton' reactors usually contain exclusively IgM and do not convert to IgG (De Clercq, 1998). IgM/IgG isotype-specific ELISAs are also helpful in assessing the time of infection in the pig or on the infected premises. The presence of IgM, alone or together with IgG, is evidence of recent infection and indicative of virus shedding, while detection of IgG alone suggests an older exposure to infection (Brocchi *et al*., 1995).

a) Virus neutralisation (the prescribed test for international trade)

The quantitative VN microtest for antibody to SVD virus is performed using IB-RS-2 cells (or suitable susceptible porcine cells) in flat-bottomed tissue-culture grade microtitre plates.

Virus is grown on IB-RS-2 cell monolayers and stored at –20°C after the addition of an equal volume of glycerol. SVD virus has been found to be stable under these conditions for at least 1 year. The sera are inactivated at 56°C for 30 minutes before testing. A suitable medium is Eagle's complete medium/LYH with antibiotics.

The test is an equal volume test in 50 µl volumes:

i) Starting from a 1/4 dilution, sera are diluted in a twofold dilution series across the plate, two rows of wells per serum and a volume of 50 µl.

ii) Previously titrated virus is added; each 50 µl unit volume of virus suspension contains about 100 $TCID_{50}$ (50% tissue culture infective dose).

iii) Controls include at least a weak positive serum and a negative serum, a cell control, a medium control and a virus titration used to calculate the actual virus titre used in the test.

iv) Incubate at 37°C for 1 hour with the plates covered.

v) A cell suspension at 10^6 cells/ml is prepared in medium containing 10% bovine serum for cell growth. 50 µl of cell suspension is added to each well.

vi) Plates are sealed with pressure-sensitive tape and incubated at 37°C for 2–3 days. Alternatively, the plates may be covered with loosely fitting lids and incubated in an atmosphere of 5% carbon dioxide at 37°C for 2–3 days.

vii) Microscopic readings are feasible after 48–72 hours; the plates may be finally fixed and stained ~~routinely~~ on the third day. Fixation is effected with 10% formalin/saline for 30 minutes; staining is done by immersion in 0.05% methylene blue in 10% formalin for 30 minutes. The plates are rinsed in

tapwater. Positive readings are blue-stained cell sheets (where the virus has been neutralised and the cells remain intact), whilst empty wells (where virus has not been neutralised) are read as negative.

viii) ~~Positive results are blue stained cell sheets; the negative wells are empty. Titres are expressed as the final dilution of serum present in the serum/virus mixture at the 50% end-point. The test is considered to be valid when the amount of virus actually used per well is between $10^{1.5}$ and $10^{2.5}$ $TCID_{50}$, and the positive standard sera are within twofold of their expected titre.~~

ix) Interpretation of the results: ~~At the Pirbright, OIE Reference Laboratory for SVD (see footnote 1), VN titres less than or equal to 1/11 are considered to be negative. Titres of 1/16 to 1/32 are doubtful and VN titres of 1/45 or more are regarded as positive. However,~~ The test is considered to be valid when the amount of virus actually used per well is between $10^{1.5}$ and $10^{2.5}$ TCID50, and the positive standard sera are within twofold of their expected titre. Titres are expressed as the final dilution of serum present in the serum/virus mixture where 50% of wells are protected. ~~As titres depend on the cell system used,~~ Laboratories should establish their own ~~criteria~~ cut-off titres by reference to both results from a negative population and standard reagents available from the OIE Reference Laboratories, in particular the low-positive serum defining the lowest level of antibodies that Laboratories should consistently score positive.

b) Enzyme-linked immunosorbent assay

In the ELISA developed by Brocchi *et al.* (1995), the SVD viral antigen is trapped to the solid phase using the MAb 5B7. The ability of test sera to inhibit the binding of peroxidase-conjugated MAb 5B7 to the trapped antigen is then evaluated. Finally, the amount of conjugated MAb bound is detected by the addition of substrate and chromogen.

i) ELISA plates are coated with 50 µl/well of MAb 5B7 at a saturating dilution in carbonate/bicarbonate buffer, pH 9.6, by overnight incubation at 4°C.

ii) The plates are washed three times with PBS containing 0.05% Tween 20, and 50 µl of SVD antigen (SVD virus grown in IB-RS-2 cells, clarified, filtered and BEI-inactivated) at a predetermined optimal dilution, is added to each well. The optimal dilution of antigen is determined by checkerboard titrations of antigen and conjugated MAb that define the working dilution giving an absorbance on the upper part of the linear region of the antigen titration curve (between 1.5 and 2.0 optical density units). Plates are then incubated for 1 hour at 37°C.

iii) After three additional washes, 50 µl of diluted test sera (inactivation is irrelevant) and control sera are incubated with the trapped antigen for 1 hour at 37°C. Sera can be tested at a single dilution (1/7.5) or titrated. In the latter case, three-fold dilutions of sera are obtained directly in ELISA wells by adding 10 µl of serum to 65 µl of buffer (1/7.5 dilution) then transferring 25 µl to sequential wells containing 50 µl of buffer, mixing, and finally discarding 25 µl. For spot-test, the screening dilution 1/7.5 is obtained by adding 7µl of each test serum (and control sera) to 45 µl of buffer previously distributed into wells.

iv) After incubation for 1 hour, 25 µl of an optimal dilution of peroxidase-conjugated MAb 5B7 (see step ii above) is added to each well and the plates are incubated at 37°C for a further 1 hour.

v) After a final series of washes, the colorimetric reaction is developed by distributing 50 µl per well of the substrate solution (for example 0.5 mg/ml orthophenylene-diamine in phosphate/citrate buffer, pH 5, containing 0.02% H_2O_2).

vi) The reaction is stopped after 10 minutes by adding 50 µl of 2N H_2SO_4. The absorbance is read at the appropriate wavelength using a microplate reader.

Antigen, sera and conjugate are diluted in PBS, pH 7.4, containing 0.05% Tween 20 and 1% yeast extract; the dilution buffer for sera contains, in addition, 1.0% mouse serum (or alternatively another source of murine immunoglobulins) to prevent nonspecific binding of pig serum to MAb 5B7 either coated to the plate or conjugated to peroxidase.

vii) *Controls:* Four wells on each plate containing all reactants except test serum confirm the maximum absorbance reading for the antigen; ~~convalescent pig serum at four selected dilutions;~~ negative pig serum; a low positive standard pig serum; optionally, a strong positive pig serum at four dilutions, previously calibrated in order to give ≥50% inhibition (see step viii below) at the highest dilution.

viii) Interpretation of the results: Reactions are expressed as the percentage inhibition by each test serum of the MAb reaction with the SVD antigen. Sera are considered to be positive when producing an inhibition ≥80% at the 1/7.5 dilution; negative when producing an inhibition <70% at the 1/7.5 dilution; doubtful when producing an inhibition ≥70% and <80% at the 1/7.5 dilution. The second dilution (1/22.5) provides an indication of the level of antibodies: strongly positive sera show >80% inhibition at both 1/7.5 and 1/22.5 dilutions, while sera registering >80% inhibition at the 1/7.5 dilution but <50%

inhibition at the 1/22.5 dilution are considered to be low positive or borderline. All positive, borderline and doubtful sera should be confirmed using the VN test.

STANDARD REFERENCE SERA FOR SVD SEROLOGY

The OIE Reference Laboratory, Pirbright, UK maintains a panel of reference sera that have been extensively validated by the National SVD Reference Laboratories of the Member States of the European Union. This panel includes the low-positive serum defining the lowest level of antibodies that should consistently provide a positive result by ELISA and Virus Neutralisation (RS01-04-94 or equivalent). Positive sera equivalent to these reference standards are also available at the OIE Reference Laboratory in Italy. MAb 5B7 is available from the OIE Reference Laboratory for swine vesicular disease in Italy.

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

No commercial SVD vaccines are currently available. ~~Standard sera can be obtained from the OIE/FAO WRL for FMD (see footnote 1). MAb 5B7 is available from the OIE Reference Laboratory for swine vesicular disease in Italy (see Table given in Part 3 of this *Terrestrial Manual*)~~

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NB: There are OIE Reference Laboratories for Swine vesicular disease
(see Table in Part 4 of this *Terrestrial Manual* or consult the OIE web site for the most up-to-date list:
http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/ http://www.oie.int/).
Please contact the OIE Reference Laboratories for any further information on
diagnostic tests and reagents for swine vesicular disease

CHAPTER 2.9.8.

MANGE*

SUMMARY

Mange is a contagious skin disease, characterised by crusty, pruritic dermatitis and hair/feather loss, and caused by a variety of parasitic mites burrowing in or living on the skin. Some alternative historical names for mange are 'la gale' (*in French*)*, 'itch', 'scab', and 'scabies'* (*a term that should be reserved only for mange caused by* Sarcoptes scabiei)*. Specifically, on domestic hosts* (*i.e. livestock, poultry, companion and laboratory animals*)*, about 50 mite species in 16 families and 26 genera may cause mange. A number of other skin conditions* (*e.g. dermatitis, wheals, blisters, nodules*) *may be confused with mange and must be considered in differential diagnoses, including those resulting from allergic reactions to other kinds of mites, various arthropod bites, fungal diseases, or reactions to physical or chemical aspects of plants. Mange diagnosis in domestic animals is based on clinical manifestations and the demonstration of mites or their developmental stages in host skin scrapings.*

Identification of the agent: *Mange mites are mostly weakly sclerotised, slow-moving, very small* (*100–900 µm*)*, and live permanently on their hosts. Although the Acari is an extremely diverse and ubiquitous group of arachnid arthropods, all of the major mange mite species fall within only two acariform lineages, the Astigmata: Psoroptidia and the Prostigmata: Rhaphignathina. Some economically important mange mite genera are* Cheyletiella, Chorioptes, Demodex, Knemidokoptes, Notoedres, Otodectes, Psorobia, Psoroptes, *and* Sarcoptes*. Specialised illustrated diagnostic keys, taxonomic descriptions, and reference specimens should be consulted to properly identify the causative agents of mange. Special collecting techniques and compound microscopy usually are necessary for diagnosis. Certain identifying characteristics of each of the mange mite groups are highlighted in the following discussion. Although availability is limited, serodiagnostic tests have been developed for certain mange mites and are useful in some circumstances.*

Requirements for vaccines ~~and diagnostic biologicals~~: *Currently, no commercial vaccines against mange are available.*

A. INTRODUCTION

Mange is a contagious skin disease, characterised by crusty, pruritic dermatitis and hair/feather loss, and caused by a variety of parasitic mites burrowing in or living on the skin. The French term for mange is 'la gale' (Pangui, 1994), and in English, it has been called 'itch', 'scab', or 'scabies' (a term that should be reserved specifically for mange caused by *Sarcoptes scabiei*).

Numerous species of mites cause mange in literally hundreds of species of wild and domestic birds and mammals. In fact, approximately 60 mite families have members that live in or on the skin, hair, or feathers of homoeothermic vertebrates and are potential mange mites. Specifically, on domestic hosts (i.e. livestock, poultry, companion and laboratory animals), about 50 mite species in 16 families and 26 genera may cause mange. Humans are host to the readily transmitted *S. scabiei*, and human scabies occurs most frequently in elderly nursing homes and children's day-care centres. Some other mange mites may cause transient disease in humans, but infestations seldom persist.

Mites (Acari) are an extremely diverse, abundant, and ubiquitous group of arachnid arthropods with about ~~50~~ 55,000 described species. Higher-level acarine classification is still an unsettled construct, but the following is a consensus system (Bochkov & Mironov, 2011; Krantz & Walter, 2009) encompassing the mange mites. Acari comprises ~~three~~ two major evolutionary lineages, ~~Opilioacariformes~~, Parasitiformes and Acariformes, but only certain acariform mites cause mange in domestic animals. ~~Two~~ Moreover, both lineages – Trombidiiformes and Sarcoptiformes – within the Acariformes contain mange mites. Trombidiiformes includes the major suborder Prostigmata, with multiple superfamilies and many families, five of which contain mange mites. Sarcoptiformes

contains ~~two mite~~ the major suborder Oribatida, ~~and Astigmata,~~ with many cohorts, superfamilies, and families included, but only 11 ~~astigmatan~~constituent families in Astigmata: Psoroptidia contain mange mites.

Some other mites may cause less serious dermatitis in animals or humans (Yunker, 1964). Certain Parasitiformes (order ~~Gamasida~~ Mesostigmata: e.g. *Ornithonyssus*, *Dermanyssus*) and other Prostigmata (e.g. *Trombicula* [and other chiggers], *Pymotes*) transiently bite a host while feeding, leaving itchy welts and wheals behind. Stored-products, animal-nest, and house-dust mites (e.g. *Acarus*, *Glycyphagus*, *Dermatophagoides*) may cause contact dermatitis (e.g. baker's itch, grocer's itch) but no persistent infestation. Certain free-living bird-nest mites (Hypoderatidae) have a parasitic nymphal stage (hypopus) that characteristically lives subcutaneously in the bird host (e.g. *Hypodectes propus* in domestic pigeons), causing skin irregularities. Pediculosis or certain fungal diseases, such as ringworm, can cause alopecia and crusty dermatitis, and even the physical (e.g. awns, urticarial hairs) or chemical (e.g. urushiol) aspects of some plants may cause host skin reactions that could be confused with mange.

B. DIAGNOSTIC TECHNIQUES

Mange diagnosis in domestic animals is based on clinical manifestations and the demonstration of mites or their developmental stages in host skin scrapings (Kettle, 1995). It is typified by hair/feather loss, crusty or scaly skin lesions, dermatitis, thickened skin, scurf, and pruritus.

1. Detecting the agent

Hair/feather loss and crusty or scaly skin are the most apparent clinical signs of mange. A number of other diseases must be considered when one is confronted with a possible case of mange, including fungi, bacteria, insect bites, irritating plants, mechanical abrasion, etc. In most cases, scrapings should be taken from the edge of the lesion, from obviously pruritic locations, and from where there are thick, crusty flakes. Take a skin scraping by holding a scalpel blade or other sharp instrument at a right angle to the skin and scraping off the outer surface of the skin. For those mite species that burrow into the skin, the scraping must be deep enough to cause a small amount of blood to ooze from the scraping site. A drop of mineral oil or glycerol ~~such as glycerine~~ may be placed on the blade to help hold the skin scrapings during the procedure. Skin scrapings should be placed in sealed containers (e.g. clean, empty salve tins; stoppered glass/plastic test tubes; small, sealable plastic bags) and promptly taken or sent to a laboratory for more thorough examination. An even more effective method of collecting mites from the skin surface and hair is by using a vacuum cleaner fitted with an in-line filter (Klayman & Schillhorn van Veen, 1981). The material collected, along with the filter, is then examined as a skin scraping would be. An otoscope can be valuable in revealing the presence of ear mites. A cotton-tipped applicator can be used to swab the ear canal if ear mites are observed or suspected; examine it in the same way as a skin scraping.

Perform an initial examination of the skin scraping under a dissecting microscope. Obviously visible mites, especially those that are alive and moving, may be picked up with a dissecting needle dipped in glycerine or a mounting medium and transferred to a drop of mounting medium on a glass slide. When the desired number of mites has been collected, gently place a cover-slip on the drop of mounting medium, taking care to avoid air bubbles. Hoyer's medium, Berlese's fluid, Vitzhum's fluid, and Heinze's modified PVA medium are all acceptable mounting media. If permanent mounts are desired, allow the slides to dry for at least 1 week at room temperature, then ring the cover-slips with nail polish or other sealant to keep them from drying out.

Mites that are embedded in oil and exudate, *Demodex* for example, may be demonstrated by placing a small amount of skin scraping directly on the slide with some glycerine or immersion oil and pressing a cover-slip on top of it. The slide then can be examined directly with a compound microscope.

Skin scrapings that contain dead mites, large amounts of skin flakes or scabs, or large amounts of hair should be processed further. Place the skin scraping (up to several grams of skin and hair) in a suitably sized beaker, then add sufficient 10% potassium hydroxide to immerse the sample. Cautiously bring the solution to a gentle boil, stirring frequently (a laboratory hot-plate with a magnetic stirrer works well for this), for 5–10 minutes or long enough to digest most of the hair and skin. This step should be performed under a chemical fume hood to limit exposure to caustic fumes. Do not boil for an extended period of time, or the mites may disintegrate. Transfer the digested material to suitable test tubes, and centrifuge at 600 ***g*** for 10 minutes. Decant the supernatant. Resuspend the pellet in a small amount of flotation medium (e.g. Sheather's solution or a mixture of 50% corn syrup and 50% water); then, fill the tube completely with flotation medium, and place a cover-slip on top of the tube, assuring that it makes contact with the flotation medium. Let stand for 1 hour, or centrifuge for 10 minutes. Carefully remove the cover-slip by lifting straight up, so that a drop of fluid remains on the underside of the cover-slip, and place on a glass slide. Any mites in the sample will have floated to the top and will be found in the drop of fluid attached to the cover-slip. Another simpler but satisfactory technique, that is used in many laboratories, is to re-suspend the pellet in a small amount of distilled water, drop onto a large (76 × 51 × 1 mm) glass slide and

cover with a 40 × 50 mm cover-slip. This is examined under a dissecting microscope (×40 or ×100) with understage lighting. The slide then may be examined under a compound microscope for the presence of mites.

DNA of *Sarcoptes scabiei* has been successfully amplified and detected by polymerase chain reaction (PCR) from human cutaneous scales (Bezold *et al*., 2001). This technique holds promise as an additional procedure for detecting specific, hard-to-find mange mites in skin scrapings.

In cases where mites are difficult to find in skin scrapings from small domestic pet animals, they sometimes may be demonstrated by faecal flotation.

2. Identifying the agent

Mange mites are mostly weakly sclerotised, slow-moving, very small (100–900 µm), and live permanently on their hosts. The general life cycle of mange mites is brief (1–5 weeks) and includes four stages: egg, six-legged larva, eight-legged nymph (one or more instars), and eight-legged adult (male and female.) Specialised illustrated diagnostic keys (e.g. Baker *et al*., 1956; Bochkov, 2010; Gaud & Atyeo, 1996; Giesen, 1990; Kettle, 1995; Klompen, 1992; Krantz & Walter, 1999; Yunker, 1973), taxonomic descriptions, and reference specimens should be consulted to properly identify the causative agents of mange. However, certain diagnostic characteristics of each of the mange mite groups are highlighted in the following discussion.

Mange in domestic animals results from the host's physiological, immunological, and behavioural responses to infestation by certain mites in any of eleven families of Astigmata or five families of Prostigmata.

a) Astigmata

Astigmatan mange mites are generally small, globose or oval in outline, and thin-skinned. The somatic cuticle often shows a pattern of fine, parallel striations (finger print patterns), with distinctively shaped and placed setae, spines, pegs, or scales, and sometimes, lightly sclerotised plates or shields. Adults usually have eight legs and anterior mouthparts that include paired palps and chelicerae used for cutting and feeding. The legs attach proximally to the body through distinct cuticular epimeres (coxal apodemes) and terminate distally in a variety of setal forms or in a pretarsal empodium that may be shaped like either a claw or a bell-like sucker (caruncle or ambulacrum.) Astigmatan mites do not have true, paired pretarsal claws. Males sometimes bear somatic suckers or other secondary sexual characteristics used in mating, but the form and placement of setae and empodia on the legs is usually sufficient to separate the sexes as well as identify the various mange mite species. Fertilised eggs are simple, soft, and translucent ovoids that are produced by mated females through a usually midventral ovipore.

i) *Sarcoptidae*

Sarcoptid mites are all obligate, burrowing skin parasites of mammals, with over 100 described species (Klompen, 1992; Bochkov, 2010). Survival time under moderate conditions for mites off the host is limited to about 10 days or less ~~(Arlian, 1989)~~. Because of their activities in the epidermal layers of the skin, mange caused by these mites is generally more severe than that caused by mites dwelling above the surface of the skin. The body outline of sarcoptids is generally rounded, dorsoventrally flattened, and the cuticle is striated. The palps are one-segmented, and the legs are usually short. Three genera contain domestic animal parasites of interest.

Sarcoptes scabiei

This mite causes sarcoptic mange (scabies) in humans and other mammals ~~(Arlian, 1989)~~. It is among the most common, widespread, and serious types of mange extant. More than 100 known species of infested hosts occur worldwide in at least 10 mammalian orders and 26 families (Bornstein *et al*., 2001). Domestic hosts include camels, cattle, dogs, sheep, goats, horses, swine, llamas, and alpacas. Sarcoptic mange in dromedaries is a particularly debilitating chronic condition with high morbidity, and it may predispose afflicted hosts to other infections. Fain (1968) suggests that humans were the original host of *Sarcoptes*, and all other hosts were secondarily infested. Despite some dissention ~~(Kutzer, 1970)~~, current scientific consensus generally ~~has followed Fain (1968) in viewing~~ views all *Sarcoptes* mites on all hosts as no more than host-adapted variants of a single, variable species. Transmission between individuals within a host species or genus may occur easily by close contact, but taxonomically unrelated hosts are not readily infested or infestations are self-limiting. For example, *S. scabiei* var. *canis* easily transfers among dogs and can move to foxes, coyotes, and other canids ~~(Samuel, 1981)~~, but humans serve as no more than transient hosts for this variant ~~(Estes *et al*., 1983)~~. Recent molecular analyses support the conspecificity of all *Sarcoptes* variants (Zahler *et al*., 1999), and an immune response has been demonstrated in Sarcoptes infested hosts (Arlian *et al*., 1994).

Mature female *S. scabiei* are approximately 500 µm long, with fingerprint-like striations on the cuticle, short and stubby legs, various characteristic setae and pegs, and with a dorsal patch of tooth-like spines. Males are similar but smaller (about 275 µm), and the tooth-like spines are reduced in size and number. The anus is posterior in both sexes, and the first pair of epimeres is fused in a midventral Y-shape. Long-stalked, unjointed pretarsal suckers occur on legs I and II in both sexes and on legs IV in males. The remaining legs all terminate in long, hair-like setae. In addition, each tarsus bears at its tip one or two highly modified setae in the form of short spurs. Nymphs resemble females but are smaller and lack an ovipore. Larvae are similar but smaller still and have only six legs.

Trixacarus caviae

This mite is a specific parasite of captive and laboratory guinea-pigs, *Cavia porcellus*, but it never has been found on wild-caught animals (Klompen, 1992). Although these mites are a little smaller, the morphology and life cycle are similar to *S. scabiei*. However, all dorsal setae in *T. caviae* are long and hair-like, unlike some of those in *Sarcoptes*, which are short and broad or peg-like; males of *Trixacarus* also lack pretarsal suckers on the fourth pair of legs, and the pedicels (stalks) of all suckers are a bit shorter than those typical of *Sarcoptes* mites. This mite may cause a severe mange in host animals, especially in the laboratory setting. A similar mite, *T. diversus*, rarely occurs on laboratory rats.

***Notoedres* spp.**

Notoedres is a large genus comprising some ~~40~~ 45 species, most of which are associated with bats (Chiroptera) (Klompen, 1992). Four species are of some concern with respect to notoedric mange in domestic animals. The cat mange mite, *N. cati*, is a cosmopolitan parasite of domestic cats, but it also infests several wild cats (e.g. bobcat, cheetah, serval, snow leopard), palm civets, coatimundis, mongooses, and domestic rabbits. These are highly contagious mites, and they cause intense mange, especially about the host's head and sometimes spreading to the legs, genital area, or even the tail. Laboratory rats are hosts to *N. muris*, which burrows into the stratum corneum and causes thickening and cornification of the skin on the pinnae, eyelids, nose, and tail. Additional hosts include other *Rattus* spp., several other rodents, two marsupials, and a hedgehog (Klompen, 1992). The laboratory mouse may be infested by two *Notoedres*, *N. musculi* and *N. pseudomuris*, but the latter primarily occurs in wild populations of this host. Each mite also infests a few other murid rodent species. The mange caused is similar to that caused by *N. muris* in rats. *Notoedres* mites are generally similar to *Sarcoptes* but about half the size, and they lack the mid-dorsal field of tooth-like cuticular spines and peg-like setae, which may be replaced by a slight scale-like pattern in the cuticular striations and short, stout setae. The anus is posterodorsal, the first pair of epimeres is not fused medially, and the tarsi of legs I and II each end in three or four short, spur-like setae, not just two.

ii) *Psoroptidae*

Psoroptid mites are obligate parasites of mammals. They dwell and feed on the surface of the host's skin. Survival time for some of these mites off the host may be two weeks or more ~~(Otranto *et al*., 2004)~~. The generally oval-shaped body is dorsoventrally flattened, has a striate cuticle with scattered setae but no spines, and bears longer legs and more prominent mouthparts than those of sarcoptid mites. The anus is posteroventral. Males usually each have a pair of terminal posterior lobes bearing diagnostic setae and a pair of ventral adanal suckers used in mating. The first pair of epimeres is not fused medially. ~~More than~~Fifty species in about 30 genera of psoroptid mites are known from at least ~~seven~~ 11 mammalian orders, with the greatest number on primates (~~O'Connor, 1982~~Bochkov, 2010). Three genera have veterinary importance for domestic animals.

Psoroptes ovis

For decades ~~following Sweatman (1958)~~, conventional practice among acarologists has been to distinguish several species of *Psoroptes* among the mites that cause psoroptic mange worldwide in wild and domestic ungulates and rabbits, e.g. *P. cuniculi* in the ears of rabbits and various ungulates, *P. equi* on the bodies of English equids, *P. ovis* on the bodies of sheep and other ungulates (Bochkov, 2010). Distinctions between the species were based primarily on host and anatomical site infested and on morphology of the males. Recently, several workers have invalidated these criteria and used genetic analysis to show conspecificity of the traditionally different species ~~(Bates, 1999; Ramey *et al*., 2000; Zahler *et al*., 1998; 2000)~~. The earliest published description for *Psoroptes* mites is that for *P. ovis*, making this the proper designation for all such mange mites on all domestic hosts. Thus, the nomenclatural situation in *Psoroptes* becomes similar to that in *Sarcoptes*, with one morphologically and genotypically variable species occurring worldwide, albeit on a smaller spectrum of hosts and with a bit less stringent host specificity among the variants. Two other named *Psoroptes* spp. remain as tentatively valid taxa occurring only on wild mammal hosts (Bochkov, 2010). Psoroptic mange in both sheep and cattle seems to vary in its severity according to the variant of *P. ovis* present, with the most severe form being a reportable condition caused by an especially virulent genotype and known as 'sheep scab'. This form has been eradicated from the USA, New Zealand, Canada, and Australia, although it still persists in many other parts of the world. Thus, particularly for further eradication efforts against psoroptic sheep mange, genotypic analysis of the involved mites may be an especially valuable tool (Falconi *et al*., 2002).

Mature female *Psoroptes* are 550–750 µm long, with a striate cuticle and four long and 16 short dorsal somatic setae ~~(Sanders *et al*., 2000; Sweatman, 1958)~~. A noticeable anterodorsal cuticular plate is present behind the mouthparts, and the midventral ovipore is an inverted U-shape. Males are about one-fourth smaller, and they have an additional, larger posterodorsal cuticular plate, a pair of posteroventral adanal suckers, and two terminal posterior lobes, each equipped with four setae of varying lengths and structures. Nymphs and larvae are somewhat similar to adults but progressively smaller, and all *Psoroptes* are pearly white in colour. In all stages, the anterior two pairs of legs are thicker and more robust than the posterior pairs, which are thinner, and in the male, shortened in the fourth pair. Legs I and II terminate in pretarsal empodial suckers on long, segmented pedicels in both sexes, with similar structures on legs IV of the female and legs III of the males. The female's third tarsus ends in two long, whip-like setae, and the male has a single short seta on tarsus IV, plus a long, thin seta accompanying the empodial sucker on tarsus III.

***Chorioptes* spp.**

This genus currently comprises five putative species of obligate ectoparasitic mites that may cause chorioptic mange in domestic and wild mammals. Three of the species, collected rarely from wild animals, are poorly known and may not be valid entities, but *C. bovis* and *C. texanus*, primarily from domestic animals, have withstood modern biogenetic scrutiny and are accepted species (~~Essig *et al*., 1999; Zahler *et al*., 2001~~ Bochkov, 2010). A number of allegedly host-specific varieties within these species are not separable from one another (Sweatman, 1957). The two species are morphologically distinguishable only by differences in the terminal posterior lobes and setae of males (Sweatman, 1957). Chorioptic mange, also called 'barn itch,' may be the most common form of mange in cattle and horses. It is a relatively mild condition that usually is more localised and less intensely pruritic than psoroptic or sarcoptic manges. This is probably because *Chorioptes* mites are able to feed and survive on host-produced epidermal debris at the skin surface, without necessarily attacking the living parts of the host's skin. Infestations tend to concentrate on the lower portions of the host, especially the feet and legs, but may include the udder/scrotum, tailhead, and perineum. In some cases, *C. texanus* infests the host's ears (Sweatman, 1957). *Chorioptes bovis* has been known for more than 16~~0~~5 years and occurs widely on cattle, goats, sheep, camelids (mainly bactrians), and possibly domestic rabbits. *Chorioptes texanus* was not discovered until 1924, and for 50 years, it was recognised only from goats and reindeer in the USA and Canada (Sweatman, 1957). Since 1975, it has been found on European elk, *Alces alces*, and several times on cattle from Brazil, Germany, Israel, and the USA ~~(Faccini & Massard, 1976; Rosen *et al*., 1989; Zahler *et al*., 2001)~~. Based on unpublished observations by the USDA, *C. texanus* may now be the prevalent *Chorioptes* species on cattle in the USA.

Both *Chorioptes* species on domestic animals are nearly identical morphologically in all stages. The circular body is dorsoventrally flattened, with a striate cuticle, and about 400 µm long in the female; males are about one-fourth smaller, and the somewhat similar nymphs and larvae are progressively smaller yet. Dorsally, adults of both sexes have both anterior and posterior cuticular shields and a variety of mostly short, hair-like setae. Ventrally, the female ovipore is a transverse slit with a pair of trailing apodemes. The mouthparts are unremarkable, and the legs are moderately long and robust, except the fourth pair in the male are very short, and the third and fourth pairs in the female are more slender. All legs in both sexes terminate distally in empodial suckers with short, unjointed stalks, except for the female's third pair, which end in two long, whip-like setae each. The male also has a long, whip-like seta on each third leg and a pair of adanal suckers. The terminal posterior lobes of males bear five setae each. The lobes of *C. bovis* each have a nearly rectangular margin, the seta at the external angle is long and whip-like, and the two spatulate setae are moderately shorter (ca. 115 µm) and broad. The lobes of *C. texanus* are each more angulate, almost bilobed, with a very short hair-like seta at the external angle and two much longer (ca. 215 µm) spatulate setae that seem narrowed basally

Otodectes cynotis

Carnivores are the primary hosts for these highly contagious mites, which mainly infest the host's ear canals but sometimes spread to the pinnae and even beyond. Clinical signs of otodectic mange (otacariasis, 'ear canker') may include rubbing and scratching the ears, vigourous head-shaking, depression, excessive drainage, and haematoma of the ear. Worldwide, *Otodectes* is probably the most frequent mange mite infesting carnivores, both wild and domestic. In addition to companion animals (e.g. dogs, cats, ferrets), these mites also affect various farm-raised furbearers (e.g. foxes, mink) and occasionally may stray to humans. As with other mange mites, past workers often have treated *Otodectes* mites from different localities or different hosts as separate varieties, or even different species, but recent molecular and phenotypic studies conclude that the genus is monobasic ~~(Lohse *et al*., 2002)~~.

Otodectes mites have a typical psoroptid morphology and life history mirroring those of *P. ovis*. The female body is about 435 µm long and oval-shaped; the male length is about 325 µm. The female ovipore is a transverse slit with trailing genital apodemes, and bilaterally, the epimeres of the first pair of legs are joined to those of legs II. The terminal posterior somatic lobes of the male are only weakly produced, but adanal suckers are present. Each lobe bears five hair-like setae of varying lengths. All of the legs are moderately long and robust, except for the fourth pair, which is much reduced, especially in the female. Empodial suckers with very short, simple pedicels occur distally on all legs except for the posterior two pairs in females,

which each end in a pair of long setae. The third tarsus of the male also bears a pair of long, whip-like setae in addition to its ambulacrum.

iii) ~~Knemidokoptidae~~ *Epidermoptidae*

This acarine family comprises ~~seven~~ 23 genera and about ~~two dozen~~100 species of mites, particularly species in the subfamily Knemidokoptidae (six genera, 17 species), that inhabit the same microhabitats in birds that Sarcoptidae occupy in mammals (Krantz & Walter, 1999)~~(O'Connor, 1982)~~. As a result, possibly due to convergence, the morphology of the two groups is similar. The body is generally globose, with cuticular striations that are sometimes modified into patches of scale-, furrow-, or tooth-like structures. The mouthparts and legs are usually short and stubby. Pretarsal suckers may be present, incomplete, or absent on all legs, and the tarsi may terminate in one or two chitinous spurs. Somatic setae are generally few, unmodified, and quite short. Knemidokoptids have a distinctive anterior dorsal shield marked by a pair of strongly sclerotised, longitudinal, paramedial apodemes running to the base of the mouthparts. Males (but not females) also may have a median posterior dorsal shield, and their first pair of epimeres is fused into a midventral Y-shape. The first epimeres in females (and immatures) may be free or joined by a transverse apodeme into a V- or U-shape. The ovipore is a transverse slit or a three-valved, inverted Y-shape, and the anus is terminal or posterodorsal. Males may or may not have adanal suckers. Most species occur, sometimes worldwide, only on various wild birds in which they may cause clinical knemidokoptic mange; however, species in three genera are of concern for domesticated and cage birds.

Knemidokoptes mutans commonly burrows in the epidermal layers of the skin on the feet and legs of chickens, turkeys, and pheasants, causing a crusty mange known as 'scaly leg.' If untreated, lameness, distortion, or loss of digits may result. The first epimeres of female *Knemidokoptes* are free; legs I and II each have two terminal spurs, but no ambulacrum occurs on any leg; the ovipore is transverse; the anus is dorsal; and the body has a mid-dorsal patch of cuticular scales. Females are 350–450 µm, and males are less than 240 µm long. As in other knemidokoptids, legs of males are longer than those of females, and all of them terminate in a small, long-stalked sucker. A second, similar species, *K. pilae*, infests the face, cere, and legs of budgerigars, leading to a condition known as 'scaly face.' These mites are slightly smaller than *K. mutans*, and both species probably occur worldwide on their respective hosts.

Picinemidicoptes laevis infests columbid birds, including the domestic pigeon, sometimes leading to clinical mange. In females, the first epimeres are fused in a U-shape; each leg has an empodial stalk only, and legs I and II end in one spur each; the ovipore is transverse; the anus is terminal; and the dorsal cuticular striae are unbroken by scales.

Neocnemidocoptes gallinae may infest the skin of the back, head, neck, abdomen, and upper legs of chickens, geese, and pheasants, causing intense pruritus. Feathers in these areas may fall out, break, or be plucked by the host, leading to a condition known as 'depluming itch.' Affected skin, especially on the neck, may become scaly, thickened, and wrinkly. Although depluming itch is less common worldwide than scaly leg, it may be more damaging and even fatal. Female mites are 340–440 µm long, but males subtend about 210 µm. The first epimeres of female *Neocnemidocoptes* are free; the tarsi each end an empodial stalk only, and one spur terminates each of the anterior two pairs of legs; the ovipore is transverse; the anus is dorsal; and the dorsal somatic cuticle is transversely striate but without scales. Two other, smaller *Neocnemidocoptes, N. columbicola* and *N. columbigallinae*, infest columbiform birds in limited circumstances and possibly might cause pathology in domestic pigeons.

iv) Miscellaneous families

Eight remaining astigmatan fur and feather mite families contain a variety of mange mites that are generally of minor significance due to their limited host ranges or relatively mild clinical effects on their hosts.

Three families of mammal parasites are worthy of note. Atopomelidae comprises over 400 species in nearly 50 genera of fur mites with known hosts in ~~five~~14 mammalian orders, mostly marsupials in the Southern Hemisphere. The body plan is variable, but most are soft, slightly elongate, flattened or cylindrical, and the legs usually have some flattened segments for grasping the host's hairs to the mite's ventral surface, which often is ridged in the coxal areas of legs I and II. *Chirodiscoides caviae* probably occurs worldwide on guinea pigs, but it has been reported commonly only in Asia and Europe, where it sometimes causes severe pruritus and alopecia to laboratory animals. Listrophoridae is another family of fur mites comprising 170 species in about 20 genera found on ~~four~~nine mammalian orders, mostly rodents and mostly in the Northern Hemisphere. These are somewhat soft, elongate, cylindrical mites with various cuticular striae, spines, and punctate shields, including a sclerotised tegmen dorsally covering the mouthparts. They cling to the host hair-shaft bases by means of a pair of ridged flaps projecting ventrally from the area between the first pair of legs. *Lepoacarus gibbus* is a common listrophorid that sometimes causes mange in domestic and laboratory rabbits, and *Lynxacarus radovskyi* lives on several wild felines and the domestic cat, where mild, scurfy mange sometimes results. Myocoptidae is a nearly cosmopolitan family containing six genera and 60 species of skin-feeding, hair-clasping mites that occur on rodents~~, insectivores,~~ and marsupials. Myocoptids are generally oval-shaped and dorsoventrally flattened. The cuticle may be extensively striate,

scale-covered, or denticulate in females, whereas male cuticles are generally less ornate and more heavily sclerotised. Host hairs are grasped by robust, highly modified legs III and IV in females and legs III in males. *Myocoptes musculinus* is probably the most ubiquitous ectoparasite of laboratory mice. Infestations are usually benign, but stressed or compromised mice may suffer alopecia, erythema, pruritus, and traumatic dermatitis (myocoptic mange.) Another, smaller myocoptid, *Trichoecius romboutsi*, occasionally occurs on laboratory mice, along with *M. musculinus* or other mites, but its role in clinical mange is unclear.

Five families of mites from the skin and feathers of birds deserve mention. These are classified among 36 astigmatan mite families in three superfamilies loosely known as feather mites (Gaud & Atyeo, 1996). Thousands of species of feather mites live on or inside the feathers or skin of nearly every kind of bird worldwide in generally commensal relationships. In rare and unexplained circumstances, the commensal status of nearly any kind of feather mite may transition to that of a parasite, leading to negative consequences for the host. Some entire families of nominal feather mites (e.g. Cytoditidae, Laminosioptidae) have become true parasites with distinct associated pathologies, even mange (e.g. Knemidokoptinae). A few species in other families are more prone than is usual to cause debilitation or injury to their hosts. In the Analgidae, *Megninia cubitalis*, *M. ortari*, *M. hologastra*, and *M. ginglymura* occur on domestic chickens and may cause depluming behavior and economic losses (Gaud *et al*., 1988). *Dermoglyphus elongatus* (family Dermoglyphidae) occurs on caged canaries, and *Dubininia melopsittaci* (family Xolalgidae) occurs on budgerigars, and excessive presence of each mite species may engender depluming and associated skin lesions in the respective hosts. Members of the families Dermationidae and Epidermoptidae (Epidermoptinae) generally feed on the skin or in the feather follicles of their bird hosts, placing them very close to being parasitic. Domestic poultry are hosts to *Rivoltasia bifurcata* and *Epidermoptes bilobatus* from the two respective families, and each mite has occasionally been associated with pityriasis (epidermoptic mange) in chickens (Baker *et al*., 1956).

b) Prostigmata

With ~~nearly~~ over ~~15~~ 19,000 named species classified into approximately 130 families, prostigmatan mites as a group exhibit tremendous morphological and biological diversity, making generalisations about them difficult. However, all of the prostigmatan mange mites belong in either of two superfamilies, Cheyletoidea (comprising seven families) and Myobioidea (one family). ~~which includes only about 1000 named species in nine families.~~ Together, these eight families include nearly 1,100 named mite species, but there are hundreds of undescribed species, as well. The anterior mouthparts in this group may be variously modified by palpal segment elaboration or reduction and by basal cheliceral fusion and extension into elongate, needle-like stylets used to pierce the host's tissues for feeding. Some ~~cheyletoids~~ prostigmatan mange mites have paired, elongate, dorsal respiratory peritremes above the mouthparts. The body usually is elongate, sometimes very much so, and usually soft and thin-skinned, but sometimes with sclerotised plates. Adults usually have eight legs that vary in length and morphology according to the habits of the family, but they each usually terminate distally in a pair of pretarsal claws and a linear empodium that often is equipped with numerous sticky hairs. Proximally, the legs may articulate with simple coxal fields or sclerotised somatic apodemes. The ovipore is a longitudinal, usually mid- or posteroventral slit, whereas, the genital pore in males is dorsal and sometimes equipped with a long aedeagus.

i) *Demodecidae*

The demodecids comprise more than 150 species of parasitic mites in seven genera from hosts in 11 mammalian orders. *Demodex* is the only genus of importance for domestic hosts, and it contains at least ~~67~~ 70 named species plus many more that are unnamed and undescribed. Although other genera display their own unique features, adult *Demodex* are elongate, spindle-shaped, or vermiform mites, 250–850 µm long, that live in the host hair follicles, sebaceous glands, Meibomian glands, and occasionally in epidermal pits. They have short anterior mouthparts with two-segmented palps and retractable needle-like stylets used to puncture surrounding host tissues and feed on predigested cellular fluids. The normally four pairs of legs are usually short, stumpy, composed of three segments each, and terminate distally in paired pretarsal claws, usually with a linear empodium. Coxal fields occupy much of the anteroventral surface of the body where the legs attach. The palps or one pair of legs of some stages of some species may be greatly elongated or otherwise modified, primarily as holdfast organs. The very thin cuticle of the body and appendages is all but devoid of setae, but the opisthosoma is usually transversely striate. Befitting the confines of their narrow follicular or glandular habitats, the immature stages, including the eggs, of *Demodex* spp. are usually spindle-shaped or elongate oval, sometimes extremely so. *Demodex* species are very host specific, only rarely inhabiting more than one species of congeneric mammal host. However, it is not uncommon for a host species to harbour two to four different species of parasitic *Demodex*. Transfer between hosts occurs only by very close contact between individuals (most probably mother to neonate), making transmission between animal species or from animals to humans very unlikely. Their very thin cuticles mean that demodecids cannot survive away from their hosts for more than a few hours.

Although *Demodex* mites frequently infest the skins of 100% of the individuals of their respective host species, their presence is usually without noticeable consequence for the hosts. On occasion, because of stress or other poorly understood factors, resident mite populations explode in numbers that result in a

pathological condition known as demodectic mange. Healthy feral animals almost never suffer from demodectic mange, and laboratory or domesticated hosts are the usual victims (Nutting, 1985). Clinical signs may range from presence of small skin papules, to large nodules, to extensive hair loss. Although rare, severe or generalised cases may lead to mites invading the host circulatory system, secondary bacterial skin infection, and even death. Among domestic animals, clinical disease (sometimes called 'red mange') is most often seen in dogs (*Demodex canis* and *D. injai*), but swine, (*D. phylloides*), goats (*D. caprae*), horses (*D. caballi*), sheep (*D. ovis*), cats (*D. cati* and *D. gatoi*), cattle (*D. bovis, D. tauri,* and *D. ghanensis*), and rabbits (*D. cuniculi*) occasionally develop demodectic mange. Humans are normal hosts for two species of *Demodex* (*D. folliculorum* and *D. brevis*).

ii) *Psorergatidae*

Worldwide, fewer than 100 species of these small parasitic skin mites are described in three genera (Giesen, 1990) (treated as subgenera of *Psorergates* by some authors). Known hosts are in eight mammalian orders, mostly rodents and bats. Adult psorergatids are about 100–200 µm long, generally circular in outline, and dorsoventrally flattened. The cuticle is very thin, finely striate, and a large, punctate, lightly sclerotised shield covers most of the dorsum. The short anterior mouthparts have stylet-like chelicerae and two-segmented palps, each of which ends in a stout, claw-like seta. There are no dorsal peritremes. The four pairs of moderately long legs are radially attached ventrally, have five segments each, and terminate distally in paired pretarsal claws but no empodium. The femur of each leg often bears a sturdy, retrorse spur ventrally. Psorergatids have relatively few setae, including a few on the mouthparts, five or six pairs on the dorsal shield, one small ventral pair, one or two long pairs on posteroventral body lobes, and less than 10 on each leg. The eggs are almost round and large, nearly two-thirds the size of the mature female. They are deposited in hair follicles or in epidermal pits made by the female. Immature stages are much like adults but smaller, with only six legs for larvae and all legs greatly foreshortened. Transfer from host to host is accomplished directly by motile adult mites, which then move selectively to less-keratinised areas of the host skin, frequently about the head, neck, and the back. There, they invade the hair follicles or burrow body-sized pits into the epidermis, feed by puncturing cell walls with their stylets, and reproduce. Psorergatid mites rarely survive off the host for more than a day.

Psorergatid infestations on healthy wild hosts and most domestic animals are generally low and of little consequence. Sometimes, however, populations of a few species may explode, particularly on sheep and laboratory mice, producing psorergatic mange. Skin damage from activities of adult mites usually is mild and only slightly irritating, but their progeny, from egg nests cut into the dermis, may enlarge these pockets into fluid- or keratin-filled papular lesions that may rupture and cause inflammation and other host immune responses (Nutting, 1985). Psorergatid mange mites of concern occur in two genera, *Psorobia* (with four pairs of marginal setae on the dorsal shield) and *Psorergates* (with three pairs of such marginal setae). Infestations of *Psorobia ovis*, the sheep itch mite, are most troubling in older animals and cause the hosts to rub, scratch, and bite at the wool in the most irritated areas, giving the fleece a ragged, tufted appearance. Powdery scurf sometimes may be present, as well. The life cycle of *P. ovis* takes about five weeks, the condition spreads slowly and inconsistently through a flock, and detection of infestations often is difficult. A similar mite, *P. bos*, occurs widely on cattle, but it seems to have little pathological effect on hosts. *Psorobia cercopitheci*, from Africa (and a similar undescribed Asian species), occasionally cause mange in colonies of laboratory primates. The laboratory mouse is subject to papular lesions on the head and neck and auricular mange caused by *Psorergates simplex* (Yunker, 1973). Incidence of these mites in some mouse colonies may be as high as 80 per cent. Another *Psorergates* mite, *P. muricola*, has been found on five different rodent species, including *Mus musculus*, and *Psorergates rattus* occurs on *Rattus norvegicus*; whether either of these mites infests or damages laboratory rodents is unknown.

iii) Cheylet~~iell~~idae

This ~~is a relatively small~~ family of ~~parasitic mites (about 100 species) that, until 1970, was part of Cheyletidae, a larger family of mostly predacious prostigmatans.~~ approximately 375 species comprises mostly free-living predator mites and about 100 species parasitic on birds and mammals. The ~~species~~ parasites are arranged into approximately 15 genera, with about ~~two~~one-third of the species on mammals and the rest on birds. Although a number of the genera contain species capable of causing limited pathology in their hosts, only a few members of the genus *Cheyletiella* are of concern as mange mites on domestic animals. *Cheyletiella* mites are 300–530 µm long, elongate rhomboidal, and distinguished by a strongly striate cuticle with one (females) or two (males) large dorsal shields. A number of moderately long, simple or barbed setae occur in distinctive patterns on the body, mouthparts, and legs. The anterior mouthparts are large, with short piercing stylets and especially robust, five-segmented palps, each of which terminates in a strong, curved claw-like seta that is lined with weak, ridge-like teeth on the inner margin. Prominent M-shaped peritremes occur on the dorsal surface of the mouthparts. The four pairs of legs are long and strong, and each terminates distally in a linear empodium equipped with a double row of sticky hairs. Although almost all other cheyletiellids also have paired pretarsal claws on each leg, none occurs in *Cheyletiella*. A small sensory organ (solenidion) occurs on the middle segment (genu) of each leg I, and its shape is (statistically) distinctive for each species (Bronswijk & de Kreek, 1976). Females lay their eggs singly and

attach them to host hairs near the skin using a finely woven mass of threads. Transmission between hosts is primarily by close contact, but phoresy on ectoparasitic insects is a possibility, as well.

For many years, the identities of the various pathological *Cheyletiella* spp. were confused under the single name *C. parasitivorax* (Smiley, 1970), and these mites were mistakenly thought of as predators on other parasitic mites. However, *C. yasguri* (on dogs), *C. blakei* (on cats), and *C. parasitivorax* (on domestic rabbits) are now separately known to be the cause of mange, and any of the three may sometimes afflict humans in close contact with infested hosts, leading to severe dermatitis, pruritus, and other signs of cheyletiellosis for them, as well. The mites move easily among the host hairs on the keratin layer of the skin, periodically attaching to the surface by means of the palpal claws and puncturing cells of the epidermis with their stylets to engorge on predigested host fluids. The disease is similar in all three domestic hosts and usually is most evident on the back, shoulders, and neck. However, clinical signs are generally mild and not very distinctive or definitive. They may include scruffy hair coat, inflammation, occasional pruritus, alopecia, and almost always, hyperkeratosis. The barely visible, moving mites in the fur of the host and the abundant, powdery white scurf associated with cheyletiellic mange have engendered for it the alternative name 'walking dandruff.'

iv) *Myobiidae*

Myobiids are small (to 900 µm), soft, elongate rectangular, somewhat dorsoventrally flattened fur mites known from five orders of mammals worldwide. More than ~~400~~450 species of myobiids have been identified, at least half of them from bats. The cuticle is generally transversely striate, without sclerotised shields, and dorsally usually bears 12 to 16 pairs of setae, many of which are expanded, leaf-like, and longitudinally striate. The anterior mouthparts are small, with simple two- or three-segmented palps, cheliceral stylets, and dorsal peritremes. The legs, especially the first pair, are strong and highly modified for grasping host hairs, one or two at a time. They terminate distally in large pretarsal claws but no empodium; sometimes one of the two paired claws on a leg is greatly reduced or absent. The apparatus for clasping hairs are characteristic and consist of various combinations of modified leg segments and setae in the form of spurs, hooks, bosses, ridges, and grooved surfaces. Nymphal and larval myobiids generally resemble their respective adults except for size. Myobiid eggs are usually attached by the females with an adhesive secretion to the bases of the host's hairs. Larvae may actually enter the hair follicles to feed on host fluids issuing from punctures made with the stylets. Nymphs and adults feed at the surface of the host skin in the same way, sometimes even puncturing capillaries and imbibing blood. The life cycles of myobiids are generally brief (ca. 14 days), and the mites freely move between host individuals. Myobiid infestations on wild mammal hosts are usually low in intensity and of little consequence (Nutting, 1985), but on laboratory rodents, they frequently expand greatly and cause intense pruritus and hair loss known as myobiic mange.

Both *Myobia musculi* and *Radfordia affinis* occur on the laboratory mouse and its wild progenitor, the house mouse, and each may cause pathology in laboratory animals. The two mites are superficially similar in appearance, but differ in many minute details, the most readily observed of which is the number of pretarsal claws present on the second leg; there are two in *Radfordia* and one in *Myobia*. *Radfordia ensifera* infests the Norway rat and the laboratory rat, sometimes causing mange in the latter. Whereas, both pretarsal claws on leg II in *R. ensifera* are of equal size, the posterior claw in *R. affinis* is smaller than the anterior one.

v) Syringophilidae

Over 350 species of these very host-specific quill mites have been discovered on a wide variety of bird hosts worldwide, but ~~only a small proportion~~less than half have been named and described, while probably thousands of unknown species remain extant. The body is elongate (about 500–950 µm) and cylindrical in keeping with the infestation site within the quills of the host. The cuticle is thin, striate, and without sclerotised plates, but a variety of usually long setae arise from its surface, particularly at the posterior end. M-shaped peritremes arise above the mouthparts, which are equipped with stylets and simple, linear palps. The legs are short, stubby, and terminate distally in paired claws and haired empodia. Sclerotised epimeres occur in coxal fields I and II. While residing in the quill shafts, syringophilids puncture the quill walls with their stylets to feed on fluids from the surrounding feather follicle tissues.

Two species of quill mites from domesticated hosts sometimes occur in large numbers and cause serious irritation and severe feather loss that might be confused with knemidokoptic mange; *Syringophilus columbae* parasitises domestic pigeons and *S. bipectinatus* occurs in the quills of chickens. Modern poultry production methods that physically separate chick broods from laying hens have been very successful in breaking the chain of passage for *S. bipectinatus* from one host generation to the next, all but eliminating the depluming problem except in more traditional production settings. Two other described ~~(2001)~~ quill mites, *Picobia polonica* from chickens and ~~*Picobia polonica*~~ *P. khulkhshani* from pigeons, have not yet been associated with host feather loss.

3. Serological tests

Researchers have shown that *Sarcoptes scabiei* and *Psoroptes ovis* infestations cause measurable specific antibody responses in hosts, namely pigs, sheep, dogs, and camels (Falconi *et al*., 2002; Lower *et al*., 2001; Lowenstein *et al*., 2004); this makes possible serological detection of sarcoptic and psoroptic manges. Enzyme-linked immunosorbent assays (ELISA) that detect antibodies to *Sarcoptes* in pigs and dogs are commercially available in some countries and have been used for serodiagnosis of scabies (Lowenstein *et al*., 2004) in Sweden and Switzerland to support scabies eradication programs in swine. ~~Research efforts are currently in progress to develop a promising new immunodiagnostic assay for *S. scabiei* using recombinant antigens (Walton & Currie, 2007). ELISA techniques for serodiagnosis and management of *Psoroptes* infestations are under development and evaluation, but some issues with sensitivity and specificity remain unresolved.~~ Recombinant antibodies for *S. scabies* and *P. ovis* are commercially available, and they seem to give more consistent test results than whole mite preparations. Although the only unequivocal proof of mange is finding and identifying the offending mites~~in skin scrapings, but in the future~~ this traditional (direct) method is being augmented by better and better biochemical (indirect) methods.

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

There are no commercial vaccines for mange. Experimentally, inoculation with *Psoroptes ovis* antigen has reduced the severity of mange. This introduces the future possibility of controlling the effects of mange without the use of acaricides (Nesbet & Huntley, 2006; Smith *et al*., 2002).

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