Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2013

SECTION 1.1.

INTRODUCTORY CHAPTERS

CHAPTER 1.1.1.

COLLECTION, SUBMISSION AND STORAGE OF DIAGNOSTIC SPECIMENS

INTRODUCTION

Laboratory investigation of animal disease is critically dependent on the quality and appropriateness of the specimens collected 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 must be collected using appropriate biosafety and containment measures in order to prevent contamination of the environment, animal handlers, and individuals doing the sampling as well as to prevent cross-contamination of the specimens themselves. 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 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 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, 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 can be negatively impacted by haemolysis in the serum sample. Microbial contamination and haemolysis are significant concerns especially when obtaining blood and serum samples from post-mortem animals. Frequent causes of haemolysed serum and plasma include exposure to excessive temperatures or time delays prior to separating sera from the red blood cells, blood collection using a needle of too small gauge, or failure to remove the needle when transferring the blood sample from the collection syringe.

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

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. 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 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 avoid autolysis and overgrowth by bacterial and fungal contaminants. Ideally, freshly collected specimens are kept at a constant cool temperature from collection until processing for testing. Where such a cold chain cannot be provided fresh specimens for some test procedures can be collected into fluids such as ethylene glycol that 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. 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 specimens, with easily cleanable surfaces and trays and/or a biosafety cabinet, depending on the RA. There should be adequate and appropriate space to store specimens, either refrigerators or freezers, taking into consideration the time the specimens are to be stored. Specimen registration equipment such as computers, printers or logbooks should be available. A bar code system can be used to identify and track the specimens.

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.

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 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.

REFERENCES

ANON (1993) Removal of blood from laboratory mammals and birds. First Report of the BVA/FRAME/RSPCA/UFAW/Joint Working Group on Refinement. *Laboratory Animals,* **27**, 1–22.

FOSGATE G.T. & COHEN N.D. (2008) Epidemiological study design and the advancement of equine health. *Eq. Vet. J.,* **40** (7), 693–700.

HEM A., SMITH A.J. & SOLBERG P. (1998). Saphenous vein puncture for blood sampling of the mouse, rat, hamster, gerbil, guinea pig, ferret and mink. *Laboratory Animals,* **32** (4), 364–368.

MANN C.J. (2003). Observational research methods. Research design II: cohort, cross sectional, and case-control studies. *Emerg. Med. J.*, **20**, 54–60.

PFEIFFER D. (2010). Veterinary Epidemiology: An Introduction. Wiley-Blackwell; pp; 37–41.

WORLD FEDERATION FOR CULTURE COLLECTIONS (2010). Guidelines for the establishment and operation of collections of cultures of microorganisms, Third Edition, Revised by the WFCC Executive Board. ISBN 92 9109 043 3.

*
*

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:

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

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

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

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

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

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

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

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

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

The negative predictive value is calculated as follows:

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

Where:

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:

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

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

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

Therefore, 289 animals would have to be randomly sampled.

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

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

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

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%

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

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

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

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

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

On-line resources

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

Free Calc - 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

*
* *

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. 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.

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 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
	Flexal virus
	Francisella tularensis (cultures only)
	Guanarito virus
	Hantaan virus

---

[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.

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	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)
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)

---

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.

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**	
	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)

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

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

Some examples for Category B shipments are given below:

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

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

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

1. Packaging

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

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

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

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 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.
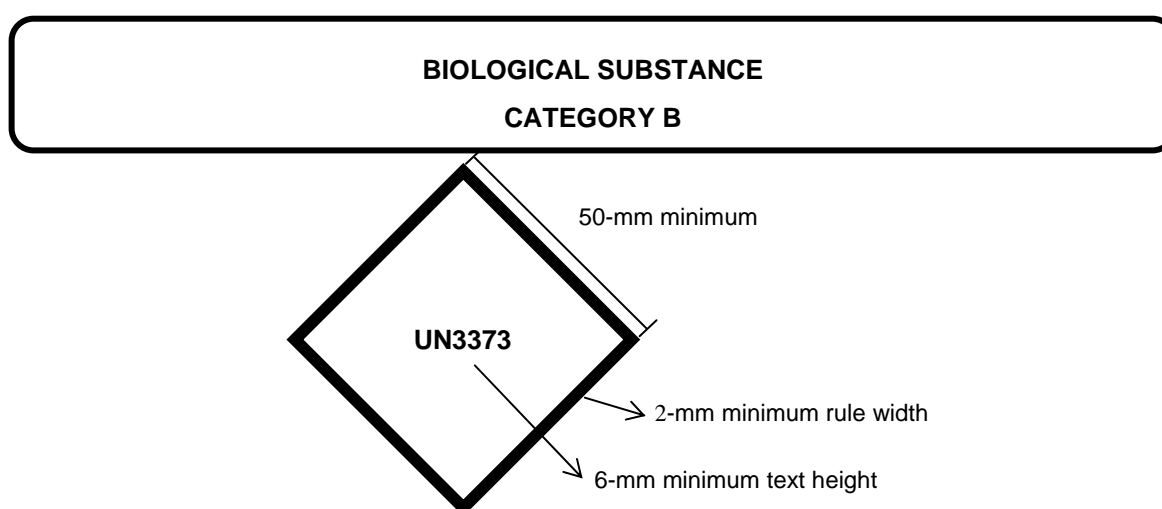

Figure 1: UN3373 mark for the transport of Category B substances

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 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. ADDITIONAL INFORMATION ON THE WORLD HEALTH ORGANIZATION, UNITED NATIONS AND OTHER INTERNATIONAL AND NATIONAL TRANSPORT GUIDANCE 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, 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/
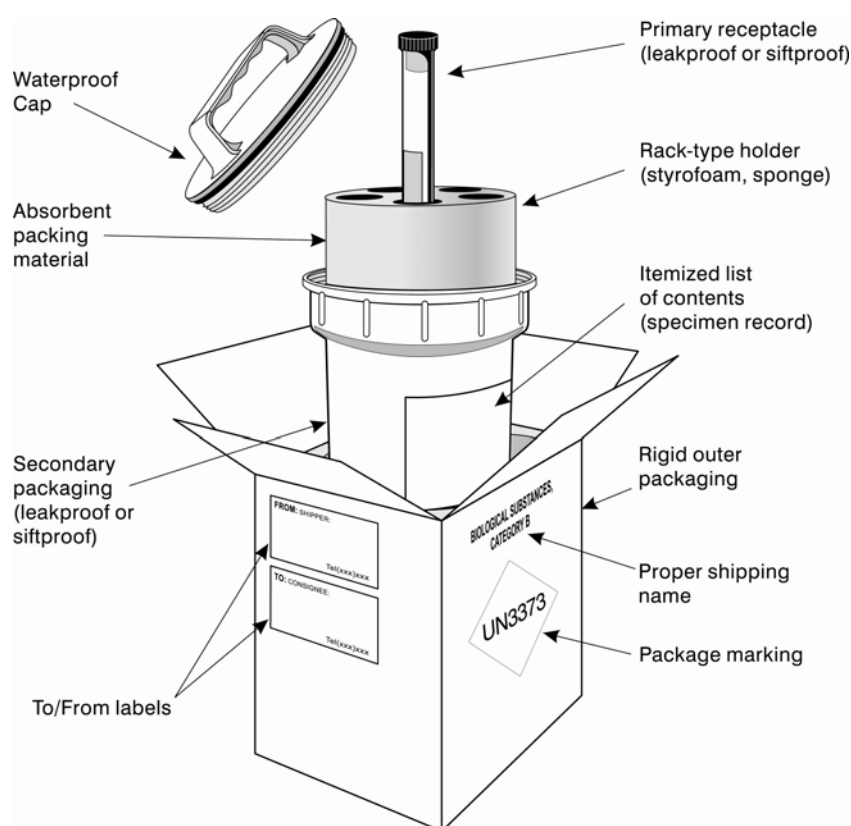
*
*   *

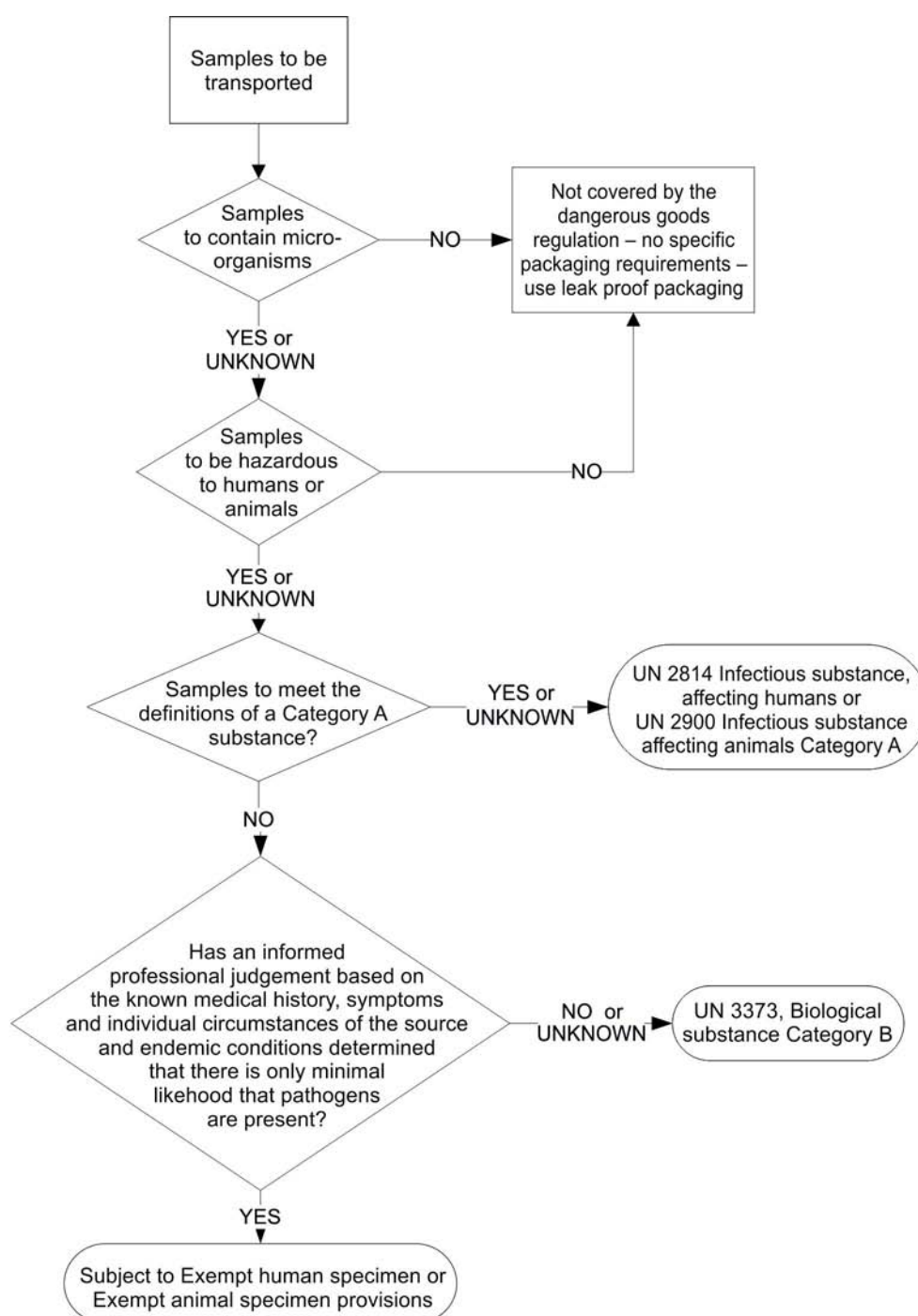A P P E N D I X   1 . 1 . 2 . 1 .

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)

APPENDIX 1.1.2.2.

DECISION TREE FOR DECIDING ON THE TRANSPORT REQUIREMENTS OF A SAMPLE

CHAPTER 1.1.3.

BIOSAFETY AND BIOSECURITY IN THE VETERINARY MICROBIOLOGY LABORATORY AND ANIMAL FACILITIES

INTRODUCTION

Laboratory work of the type described in this Terrestrial Manual *should be carried out with a minimum of risk to the health of the staff (*biosafety*) and the environment (*biocontainment*). This requires careful consideration of the risks involved in a particular procedure, followed by appropriate measures to minimise the risk of human disease and of possible release into the environment. This is a complex subject that can only be considered in outline in an introductory chapter. This chapter is concerned almost exclusively with risks from infectious agents, but physical and chemical injuries in microbiology laboratories must also be prevented. Risks from infection are reduced by good laboratory techniques and secure facilities, which aid in the containment of pathogens. It is important to understand that containment of pathogens can be used for two purposes. One is to prevent disease in humans in the laboratory; the other is to prevent the release of the pathogen into the environment and causing disease in animals or humans. Often the same methods of containment are used for both preventing laboratory-acquired infection in humans and for preventing escape of pathogens that could cause an outbreak of animal diseases. Although the methods, techniques and facilities required may be the same, the list of pathogens and categorisation into levels of risk will differ depending on whether it is human or animal diseases control that is the primary objective.*

Existing national and international reference laboratories have considerable experience in the operation of safe working practices and provision of appropriate facilities. When new laboratories are being established, it would be prudent to seek advice from the relevant regulatory authorities and the competent authorities at established institutes. It is important to comply with legislative requirements.

A. ASSESSMENT OF RISK FROM PATHOGENS

It is necessary first to assess the risk from a pathogen, so that it can be assigned to a Risk Group. A further risk assessment can be conducted, based on the proposed work, to determine the appropriate containment level. To assess the risk to humans and animals from a particular pathogen it is necessary to know whether infection with that organism can cause clinical disease and/or mortality in humans and animals, and whether it could then spread to cause disease in the general human and/or animal population. There are additional requirements related to the containment of animal pathogens and the prevention of the spread of infection to animals. To assess these risks it is necessary to know the epidemiological background of the organism and also such attributes of the organism as infectivity for humans and animals, stability in the environment, ability to infect by different routes of exposure, and susceptibility to specific treatments or prophylaxis (Acha & Szyfres, 2001; Advisory Committee on Dangerous Pathogens, 1995; Bell *et al.*, 1988; Beran & Steele, 1994). It is relatively easy to obtain this information when working with a known pathogen, but the problem is more complex in a diagnostic laboratory receiving clinical material that may be infected with a variety of unknown pathogens, some of which could be extremely hazardous to human health or pose a significant threat to animal populations. Some of the considerations to take into account when evaluating risk are:

1. Known occurrence of human and animal infection with the organism or related organisms with similar characteristics, any history of laboratory-acquired infection, infective dose and disease severity; production of toxins or allergens.

2. The volume of culture to be handled and the concentration of the organism likely to be present. (Procedures such as antigen or vaccine production that require large quantities of organisms usually carry a higher risk than attempted isolation procedures.)

3. The origin of the sample, for example samples from wildlife species may contain human or animal pathogens not normally encountered.

4. The history of the isolate being handled. Pathogens on primary isolation or of low passage level are often more dangerous than pathogens of high passage level. In some cases, pathogenicity may be enhanced by passage or subculture using different media.

5. The possibility of aerosol formation should be especially taken into consideration when handling fluid samples or, for example, during grinding, homogenisation and centrifugation.

6. The threat that the organism may pose to food-producing or companion animals or to wildlife, irrespective of the threat to laboratory personnel. Additional precautions for handling and storage are required for animal disease agents from foreign countries.

7. The physical state of the employees. For example, in the case of pregnancy, immunodeficiency or allergy, special precautions may be required. Sometimes certain individuals have to be excluded from particular types of work that would be especially hazardous to them.

8. A higher level of risk may arise when agents such as *Brucella* or *Mycobacterium* are inoculated into animals. To evaluate the impact of animal inoculation, a risk assessment should be conducted and the following factors should be considered:
   i) Host species versus inoculated species;
   ii) Strain/treatment and concentration of the inoculum;
   iii) Route of inoculation;
   iv) Animal housing;
   v) Types of sampling during the experiment.

9. Some pathogens need to be transmitted by specific vectors or require intermediate hosts to complete their life cycles before they can infect animals and cause disease. In countries where such vectors or intermediate hosts do not occur, or where climatic or environmental factors mitigate against their survival, the pathogen poses a lower risk to animal health than in countries where such vectors or intermediate hosts occur naturally or could survive.

B. GROUPING OF MICROORGANISMS BY HUMAN AND ANIMAL HEALTH RISK

The considerations outlined above have been used by several national authorities to designate microorganisms into four Risk Groups (Advisory Committee on Dangerous Pathogens, 1995; Barbeito *et al.*, 1995) representing increasing risks to human health. Such categorisation of pathogens makes no allowance for people who are particularly susceptible, for example due to pre-existing disease, a compromised immune system or pregnancy. The four Groups may be summarised thus:

- Group 1 – Organisms that are unlikely to cause human or animal disease and are disease-producing organisms in animals that are enzootic but not subject to official control.

- Group 2 – Organisms that may cause human or animal disease but are unlikely to be spread in the community or animal population and for which effective prophylaxis and treatment are available; examples of Group 2 animal pathogens:
   i) They do not depend on vectors or intermediate hosts for transmission.
   ii) There is very limited or no transmission between different animal species.
   iii) Geographical spread if released from the laboratory is limited.
   iv) Direct animal to animal transmission is relatively limited.
   v) Mode of transmission is primarily through ingestion, inoculation or mucus membrane route.
   vi) The need to confine diseased or infected nondiseased animals is minimal.
   vii) The disease is of limited economic and/or clinical significance.
   viii) Short-term survival in the environment and effective treatment or prevention is available.
   ix) May be either exotic or enzootic but are subject to official control and have a low risk of spread from the laboratory.

- Group 3 – Organisms that can cause severe human or animal disease and may spread in the community and/or animal population but for which there is usually effective prophylaxis and treatment; examples of Group 3 animal pathogens:
   i) They may depend on vectors or intermediate hosts for transmission.
   ii) Transmission between different animal species may readily occur.
   iii) Geographical spread if released from the laboratory is moderate.
   iv) Direct animal to animal transmission occurs relatively easily.

    v)   The statutory confinement of diseased, infected and in-contact animals is necessary.
    vi)   The disease is of severe economic and/or clinical significance.
    vii)   Prophylactic and/or therapeutic treatments are not readily available or of limited benefit.
    viii) Mode of transmission may be through the airborne route or direct contact.
    ix)   Are either exotic or enzootic but are subject to official control and that have a moderate risk of spread from the laboratory.

- Group 4 – Organisms that cause severe human or animal disease, may represent a high risk of spread in the community or animal population and for which there is usually no effective prophylaxis or treatment.
  i)   They may depend on vectors or intermediate hosts for transmission.
  ii)   Transmission between different animal species may occur very readily.
  iii)   Geographical spread if released from the laboratory is widespread.
  iv)   Direct animal to animal transmission occurs very easily.
  v)   Can be transmitted through casual contact or indirectly.
  vi)   The statutory confinement of diseased, infected and in-contact animals is necessary.
  vii)   The statutory control of animal movements over a wide area is necessary.
  viii) The disease is of extremely severe economic and/or clinical significance.
  ix)   No satisfactory prophylactic and/or therapeutic treatments are available.
  x)   Have a high risk of spread from the laboratory into the environment and the national animal population.

Infectious organisms that might be encountered in laboratory work have been assigned to Risk Groups 1–4 by authorities in several countries (Advisory Committee on Dangerous Pathogens, 1995; Barbeito *et al.*, 1995). Some examples of pathogens that may cause disease in humans, and also may be found in a veterinary laboratory, are listed in Table 1 Also, some very serious Group 4 agents, including Hendra and Nipah, have been isolated from diagnostic specimens in veterinary laboratories.

Table 1. *Examples of some of the microorganisms in Risk Groups 2 and 3 that are capable of causing human disease and that may be present in a veterinary laboratory*

Group 2

Viruses: Influenza viruses types A, B, C other than notifiable avian influenza (NAI); Newcastle disease virus; Orf (parapox virus)

Bacteria: *Alcaligenes* spp.; *Arizona* spp.; *Campylobacter* spp.; *Chlamydophila psittaci* (nonavian); *Clostridium tetani; Clostridium botulinum; Corynebacterium* spp.; *Erysipelothrix rhusiopathiae; Escherichia coli; Haemophilus* spp.; *Leptospira* spp.; *Listeria monocytogenes; Moraxella* spp.; *Mycobacterium avium; Pasteurella* spp.; *Proteus* spp.; *Pseudomonas* spp.; *Salmonella* spp.; *Staphylococcus* spp.; *Yersinia enterocolitica; Yersinia pseudotuberculosis*

Fungi: *Aspergillus fumigatus; Microsporum* spp.; *Trichophyton* spp.

Group 3

Viruses: Rabies virus; Equine encephalomyelitis virus (Eastern, Western and Venezuelan); Japanese B encephalitis virus; Louping ill virus

Bacteria: *Bacillus anthracis; Burkholderia mallei* (*Pseudomonas mallei*)*; Brucella* spp.; *Chlamydia psittaci* (avian strains only); *Coxiella burnetti; Mycobacterium bovis*

C. REQUIREMENTS FOR WORK WITH INFECTIOUS AGENTS

1. Known pathogens

Having decided the risk level of certain work, it is then possible to decide the appropriate 'containment level' that is needed to minimise the risk of human disease and the risk of spread of disease to animals and the environment. The containment level is defined by a combination of the physical facilities and working practices employed. Organisms of the four Risk Groups indicated above may be placed into containment levels appropriate for safe working, see below. Laboratories usually appoint a Biological Safety Officer, responsible for ensuring that microorganisms are handled at the appropriate containment level. They should have sufficient expertise and be of sufficient seniority to oversee and advise on all safety matters. In large organisations with a network of laboratories, it is appropriate to appoint a central Safety Officer to advise on and coordinate safety matters of a corporate nature, which are implemented by local laboratory Safety Officers at each site. The working methods for a particular procedure or work station should be written out and readily available. Staff must be fully trained and fully aware of any health risks associated with their work and in procedures for reporting incidents or accidents. Staff should also be given a medical card indicating pathogens to which they might be exposed. In some cases, staff can be specially vaccinated to give additional protection, e.g. when working with the rabies virus; this should also be recorded on the medical card. Such information is useful for a medical practitioner in the event of illness occurring. Regular medical examinations of employees are recommended and, as appropriate, monitoring tests of employees working with the organisms that cause certain serious human diseases, such as brucellosis and tuberculosis.

Much information is available on containment of pathogens, and sophisticated apparatus and buildings may be constructed for containment of the more hazardous organisms as required by the guidelines, standards and regulations of each country. The requirements depend on the containment required, from the most basic to the highest level.

Essential requirements for all laboratory work. The essential requirements for any work with infectious agents, however innocuous they may seem, are as follows:

1. The laboratory should be easy to clean, with surfaces that are impervious to water and resistant to chemicals. There shall be a wash-hand basin and emergency shower, including an eye bath, in each laboratory suite as appropriate for the chemicals and other hazards present. Procedures shall be established for frequent cleaning and disinfection during and at the end of the work period;

2. Personnel access to the work area should be restricted; appropriate security measures such as controlled electronic access may be necessary with higher risk agents.

3. Personal protective equipment such as long-sleeved lab coats or gowns, closed-toe footwear, disposable gloves, masks, safety glasses, face shields, and oro-nasal respirators, as appropriate, shall be worn in the laboratory and removed when leaving the laboratory

4. The laboratory door should be closed when work is in progress and ventilation should be provided by extracting air from the room. (Where biosafety cabinets are used, care shall be taken to balance ventilation systems.);

5. Food (including chewing gum, candy, throat lozenges and cough drops) and/or drink shall not be stored or consumed in laboratories;

6. Smoking and/or application of cosmetics shall not take place in the laboratory;

7. Pipetting shall not be done by mouth;

8. Care shall be taken to minimise the production of aerosols;

9. Emergency response plans should be developed to deal with the biohazard of spills. Some of the items addressed in the plans should include having effective disinfectant available for cleaning spills, removal of and decontamination of contaminated protective clothing, washing of hands, and cleaning and disinfection of bench tops;

10. Used laboratory glassware and other contaminated material shall be stored safely. Materials for disposal shall be transported without spillage in strong containers. Waste material should be autoclaved, incinerated or otherwise decontaminated before disposal. Reusable material shall be decontaminated by appropriate means;

11. No infectious material shall be discarded down laboratory sinks or any other drain;

12. Any accidents or incidents shall be recorded and reported to the Safety Officer.

Containment level for Group 2 pathogens, in addition to the points given above, a Class I, II or III microbiological safety cabinet should be used when there is potential for generating aerosols or when handling large quantities of culture or where there is a real need to protect the biological product (see Section D). Appropriate signs are required at all entry doors to indicate the hazard present and the name and telephone number of the person(s) responsible. Emergency protocols should be posted within the laboratory to advise personnel of procedures to follow in case of a pathogen spill or the need to evacuate the laboratory in the event of a fire or other emergency.

Containment level for Group 3 pathogens, it is advisable that the laboratory be in an isolated location; access should be limited to appropriately trained level 3 staff. Emergency protocols should be posted within the laboratory to advise personnel of procedures to follow in case of a pathogen spill or the need to evacuate the laboratory in the event of a fire. OIE containment level for Group 3 pathogens surpasses biosafety level-3 (BSL-3) guidelines as outlined by the United States Department of Health and Human Services (DHHS) joint publication with CDC and NIH (2009) and the United States Department of Agriculture (USDA, 2002).

In addition to the previous requirements, the laboratory shall be under negative pressure and the pressure differentials should be monitored; a procedure should be developed to provide an alarm if there is a problem and personnel to respond to the alarm. A ventilation system is required that removes air from the laboratory through a high efficiency particulate air (HEPA) filter. HEPA filters shall be verified regularly (usually annually); this would include HEPA filters in biosafety cabinets and on room and equipment exhausts. The laboratory should be sealable for fumigation and contain an airlock entry. There is a requirement to treat effluent depending on the pathogen. Biological safety cabinets of Class I, II or III shall be used whenever the process to be undertaken is likely to generate an aerosol (DHHS/CDC/NIH, 2000). It may be necessary for staff to shower on exit from the laboratory and they must wear dedicated laboratory clothing that is left in the laboratory before leaving the building.

Note. Because of the link between bovine spongiform encephalopathy (BSE) and new variant Creutzfeldt-Jakob disease in humans, BSE and related agents are now categorised with the human transmissible spongiform encephalopathies in Risk Group 3. Consequently, veterinarians and laboratory workers conducting necropsies on BSE-suspect animals or handling tissues derived from such animals must conduct the work under appropriately strict containment conditions, sometimes with derogations allowed by the nature of the work and the results of local risk assessment. It is important that appropriate protective clothing be worn and that a strict code of practice be followed to prevent exposure to the agent. Laboratories conducting work on BSE must comply with national biocontainment and biosafety regulations (Advisory Committee on Dangerous Pathogens, 1998).

Containment level for Group 4 pathogens, the most stringent precautions are required, including access to the building through air locks, and the building being maintained under negative air pressure. Inlet air to the laboratory shall be filtered through a single HEPA filter and extracted air through double HEPA filters in series. All work with infective materials shall be conducted in a Class III cabinet or in a Class II cabinet in conjunction with the use of one-piece positive-pressure suits. All sewage from the laboratory, laboratory effluent and autoclave drain effluent shall be treated by appropriate means to ensure that all infectious material is destroyed before entering the sewerage outside the laboratory. Staff shall shower and change their clothing before leaving the building. Other precautions as described for Group 3 would also apply. The use of one-piece positive-pressure suits is now an internationally accepted way of providing additional protection at level 4.

OIE guidelines for the containment level for Group 4 pathogens are generally equal to the USDA's biosafety level 3 Ag guidelines (USDA, 2002). The primary difference between OIE level 4 and BSL-3 Ag is that the BSL-3 Ag guidelines specify that the laboratory will be airtight and shall pass a pressure decay test to confirm that it does not surpass the prescribed maximum leak rate.

2. Diagnostic specimens

Veterinary diagnostic centres readily receive specimens that are submitted because they are suspect for a variety of diseases. The infectious nature of the specimens is usually unknown, but they have the potential to contain biological agents that may cause disease in animals and humans. Practices and procedures need to be in place that will minimise the risk of occupational exposure of employees to such pathogens. Unless suspected of containing a pathogen requiring a higher containment level, it is advisable that initial processing of all unknown specimens should be carried out as though the material contained a Group 2 pathogen. The most important aspects are to prevent percutaneous, mucous membrane exposure, particularly inhalation and ingestion. Biological safety cabinets should be used for all manipulations that may generate aerosols. Class I or II are appropriate depending on the need for protection of the samples from contamination. Additionally, there should be no mouth pipetting, personal protective clothing shall be worn with, in some cases, eye and respiratory protection, depending on the anticipated level of exposure. Although initial diagnostic procedures may be carried out at level 2, once a Group 3 or 4 organism has been isolated (or suspected) further work must be carried out at the higher containment level.

D. MICROBIOLOGICAL SAFETY CABINETS

These are used at the different containment levels, as described in Section C above. They are of three types:

Class I: An open-fronted cabinet designed specifically to provide operator and environmental protection and not to give protection to the work being handled.

Class II: An open-fronted safety cabinet, sometimes referred to as a laminar flow recirculating cabinet. They are designed to give operator, product and environment protection.

Class III: These cabinets are closed, with glove ports at the front, and provide the highest degree of containment by complete separation of work and worker. Some cabinets have a removable glove port and are known as Class III/I cabinets, i.e. they can be used in either mode.

Descriptions of safety cabinets and safe working practices have been published (Collins, 1990; International Atomic Energy Agency [IAEA], 1994; DHHS/CDC/NIH, 2000).

E. STORAGE OF PATHOGENS

Storage of live pathogens requires appropriate containment and security to avoid risks due to breakage or unauthorised use of material. Storage facilities should be appropriately labelled to indicate the nature of the pathogens (e.g. their Group) and the contact information for the person(s) responsible for them. A complete inventory of the pathogens in storage should be kept up to date and available. Special care must be taken when

opening glass vials of freeze-dried pathogens, as these can sometimes shatter. Care must be taken when working with liquid nitrogen or rooms where asphyxiating gasses may be produced.

Many of the considerations given above relate not only to human safety but also to prevention of the spread of infection to animals. In a veterinary laboratory an important responsibility is to minimise any risk of escape of pathogens to animals, either wild or domestic, in the outside community. Close communication must be maintained with the veterinary authorities. There may be national requirements for special licences to work with certain microorganisms.

F. PHYSICAL AND CHEMICAL HAZARDS

Laboratory work involves many manipulations that are potentially dangerous, such as handling glassware and work with needles or other sharp instruments. There shall be appropriate procedures and equipment for the safe and proper disposal of needles and other 'sharps'.

Laboratory staff should be protected from the risk of receiving a burn from hot solids or liquids. Autoclaves shall be fitted with safety devices to prevent accidental opening of doors when under pressure, and be regularly serviced and tested. Heat-protective gloves, apron and face shields with brow and chin guards shall be provided. Extreme cold can also be a risk, for example when working with liquid nitrogen; splashes on exposed skin can be very damaging. Gloves should be worn that provide insulation from cold and that are also waterproof, to prevent penetration of the liquid nitrogen. Face shields with brow and chin guards and boots should also be worn when working with liquid nitrogen. Nitrogen evaporating from liquid nitrogen storage in poorly ventilated rooms can lead to depletion of oxygen with fatal consequences.

Irradiation is a serious health risk that may be present due to the use of X-ray machines, or use of gamma-emitters or other sources. Equipment shall be regularly serviced and tested. All use of radioactive material must be meticulously recorded. All staff must wear a personal radiation-monitoring device and have annual health checks. Local and national regulations must be followed (IAEA, 1994).

A wide range of chemicals are used in veterinary laboratories, many of which may be toxic or mutagenic, and some may be carcinogenic. It should be remembered that it is the dose that makes the poison. Vapours are especially hazardous, and some chemicals can be absorbed by penetration of intact skin. Steam sterilisation may make toxic chemicals volatile and endanger personnel who unload the autoclave/pressure steam steriliser. Procedures sufficient to protect pregnant laboratory workers should be followed at all times. A list of hazardous chemicals shall be maintained, and a file record kept of chemicals to which individual staff members could be exposed. This is now a legal requirement in some countries. Chemicals shall be correctly stored in appropriate containers and at the correct temperature. Those that are flammable shall be kept in a fireproof chemical store. A record must be maintained of the purchase and use of hazardous chemicals: how much, when used, by whom and for what purpose. Disposal of some chemicals is subject to official regulation.

Further information on physical and chemical safety precautions can be found in the literature (Office of Biosafety, Laboratory Centre for Disease Control, Health and Welfare Canada, 1996; Rayburn, 1990).

G. LABORATORY ANIMAL FACILITIES

Work with pathogens in laboratory animals poses special risks. Animal rooms have to be constructed to appropriate standards and containment levels, just as laboratories. Containment in animal houses is very important because of the large amount of infectious agents that they may generate. Similar considerations also apply regarding the training of staff, protective clothing and the recording of working procedures. Special care must be taken to avoid injury to staff, e.g. through animals biting and kicking or self inoculation accidents. Any such incidents must be recorded and wounds appropriately treated. There shall be provision for autoclaving steam sterilisation, incineration or rendering of carcasses and for the thorough cleansing and disinfection of animal rooms. The animal rooms should not only provide a suitable environment for the animals themselves but should be constructed and ventilated in such a way as to ensure comfort for the attending personnel. This is a large subject that can only be referred to briefly here (Barbeito et al., 1995; Canadian Food Inspection Agency, 1996). Also, an excellent book on health and safety in laboratory animal facilities is available (Wood & Smith, 1999).

H. EMERGENCY PROVISIONS

First-aid equipment should be readily available, but stored in a location that is unlikely to be contaminated by work conducted in the laboratory (for example, in the air-lock or ante-room). This equipment shall be appropriate to the

work and properly maintained. It shall be kept ready to hand for immediate emergency use by trained first aid personnel. Bandages and dressings should be available. Some staff shall receive training in safety and first aid from recognised authorities and shall possess a valid certificate as evidence of competence. Personnel working in Containment Level 4 facilities shall have advanced first aid competence. Their names and locations should be known to everyone and posted on notice boards. All staff should be aware of the importance of safety. There must be suitable procedures and equipment for dealing with spillages and decontamination. A record must be kept of all incidents and in some countries there may be a legal obligation to report incidents to the enforcing authority.

There must be written procedures for dealing with emergency failure of all safety and containment systems, for example in biosafety cabinets or biocontainment rooms, which can lead to loss of containment.

Many laboratories have a staff safety committee to increase safety awareness and to discuss safety issues with management. Personnel are responsible for their own safety and those around them. Managers are equally responsible for safety in their area of command and should not allow consideration of speed or cost of work to come before the safety of personnel or containment of animal disease agents.

There must be an emergency procedure for obtaining medical assistance if required, and for hospitalisation in appropriate infectious disease facilities when needed. Fire alarms shall be fitted, and tested regularly. The institute or laboratory must designate a warden to control and communicate in emergency situations and conduct periodic drills to make staff aware of what to do and where to assemble in the event of an emergency. The warden is responsible for checking that everyone is in a safe location. Procedures for natural disasters, such as hurricanes and earthquakes, should be in place where they present a risk. All these procedures should be written down and periodically reviewed.

I. TRANSPORT OF INFECTIOUS MATERIAL

Great care must be taken when preparing and packing diagnostic specimens, infectious materials and pathogens for transport, to ensure that there is no breakage of containers or leakage of contents that could put at risk personnel in the transport system or animals that may come in contact with contamination. Applicable local, national and international regulations for the transportation of dangerous goods (diagnostic or clinical sample and infectious materials) and importation of animal pathogens must be followed. These are summarised in Chapter 1.1.1 Collection and shipment of diagnostic specimens.

When categorising animal pathogens into specific Groups, the following criteria should be taken into account:

a)   Group 1 animal pathogens

Disease-producing organisms that are enzootic but not subject to official control.

b)   Group 2 animal pathogens

Disease-producing organisms that are either exotic or enzootic but subject to official control and that have a low risk of spread from the laboratory.

c)   Group 3 animal pathogens

Disease-producing organisms that are either exotic or enzootic but subject to official control and that have a moderate risk of spread from the laboratory.

d)   Group 4 animal pathogens

Disease-producing organisms that are either exotic or enzootic but subject to official control and that have a high risk of spread from the laboratory into the environment and the national animal population.

J. CONTAINMENT GROUPS

1.   The principal purpose of containment is to prevent the escape of the pathogen from the laboratory into the national animal population. Some animal pathogens can infect humans. In these instances the risk to human health may demand additional containment than would otherwise be considered necessary from purely animal health considerations. The risk of human to animal transmission of disease must also be considered and controlled. In addition, other animals being used for experimental work on the pathogen should be held in the appropriate containment level.

2. The level of physical containment and biosafety procedures and practices should be not less than the Group into which the pathogen has been placed and the detailed requirements should be appropriate to the type of organism (i.e. bacterium, virus, fungus or parasite). The lowest containment level will be required for pathogens in Group 1 and the highest level for those in Group 4. Guidance on the containment requirements for Groups 2, 3 and 4 is provided in Section K.

3. Arthropods may be pathogens or vectors for pathogens. If they are a vector for a pathogen being used in the laboratory, the appropriate containment level for the pathogen will be necessary in addition to the containment facilities for the arthropod.

K. GUIDANCE ON THE LABORATORY/ANIMAL FACILITY REQUIREMENTS FOR THE DIFFERENT CONTAINMENT GROUPS

Requirements of the laboratory/animal facility	Containment Group		
	2	**3**	**4**
A) Laboratory/animal facility setting and structure			
1. It is advisable that the laboratory/animal facility be in an isolated location		Yes	Yes
2. Not next to known fire hazard	Yes	Yes	Yes
3. Workplace separated from other activities	Yes	Yes	Yes
4. Personnel access limited	Yes	Yes	Yes
5. Protected against entry/exit of rodents and insects	Yes	Yes	Yes
6. Liquid effluent must be sterilised and monitored		Yes	Yes
7. Liquid effluent from steam sterilisers shall be sterilised and monitored			Yes
8. Isolated by airlock. Continuous internal airflow		Yes	Yes
9. The laboratory/animal facility shall be under negative pressure and the pressure differentials should be monitored		Yes	Yes
10. Input air to be filtered using HEPA or equivalent such as gas tight damper; exhaust air to be single HEPA filtration for laboratories and double HEPA filtration for animal facilities.		Single on extract	Single for input, double for extract
A) Laboratory/animal facility setting and structure (cont.)			
11. HEPA filters shall be verified regularly (usually annually)		Yes	Yes
12. Mechanical air supply system with fail-safe system and an alarm provided if there is a problem		Yes	Yes
13. Laboratory/animal facility sealable to permit fumigation		Yes	Yes
14. Incinerator, pressure steam steriliser or renderer for disposal of carcasses and waste	Available	Yes	Yes on site
15. The laboratory/animal facility should be easy to clean, with surfaces that are impervious to water and resistant to chemicals. There shall be a wash-hand basin and emergency shower, including an eye bath, in each laboratory suite as appropriate for the chemicals and other hazards present. Procedures shall be established for frequent cleaning and disinfection during and at the end of the work period	Yes	Yes	Yes
B) Additional Laboratory facility requirements			
16. Class I or II biological safety cabinet available	Yes	Yes	Yes

Requirements of the laboratory/animal facility	Containment Group		
	2	**3**	**4**
17. Class III biological safety cabinet available		Yes	Yes
18 HEPA filters shall be verified regularly (usually annually)	Yes	Yes	Yes
19. Direct access to autoclave/pressure steam steriliser	Yes	Yes with double doors	Yes with double doors
20. Specified pathogens stored in laboratory	Yes	Yes	Yes
21. Double-ended dunk tank required		Preferable	Yes
22. Personal protective clothing and equipment not worn outside laboratory	Yes	Yes	Yes
23. Full body shower and change of clothing required before exiting laboratory		It may be necessary for staff to shower on exit from the laboratory and they must wear dedicated laboratory clothing that is left in the laboratory before leaving the building	Yes
24. Safety Officer responsible for containment	Yes	Yes	Yes
25. Staff receive special training and demonstrate competence in the requirements needed	Yes	Yes	Yes
C) Laboratory discipline			
26. Warning notices for containment area to indicate the hazard present and the name and telephone number of the person(s) responsible	Yes	Yes	Yes
27. Emergency protocols should be posted within the laboratory to advise personnel of procedures to follow in case of a pathogen spill or the need to evacuate the laboratory in the event of a fire or other emergency	Yes	Yes	Yes
28. Laboratory must be lockable	Yes	Yes	Yes
29. Authorised entry of personnel	Yes	Yes	Yes
30. Protective clothing, including gloves, masks, eye shields, and oro-nasal respirators, as appropriate, shall be worn in the laboratory and removed when leaving the laboratory	Yes	Yes	Yes
C) Laboratory discipline (cont.)			
31. The laboratory door should be closed when work is in progress and ventilation should be provided by extracting air from the room. (Where biosafety cabinets are used, care shall be taken to balance ventilation systems.)	Yes	Yes	Yes
32. Food and/or drink shall not be stored or consumed in laboratories	Yes	Yes	Yes
33. Smoking and/or application of cosmetics shall not take place in the laboratory	Yes	Yes	Yes
34. Pipetting shall not be done by mouth	Yes	Yes	Yes
35. Care shall be taken to minimise the production of aerosols	Yes	Yes	Yes
36. No infectious material shall be discarded down laboratory sinks or any other drain	Yes	Yes	Yes
37. Used laboratory glassware and other materials shall be stored safely before disinfection. Materials for disposal shall be transported without spillage in strong containers. Waste material should be autoclaved, incinerated or otherwise made safe before disposal. Reusable material shall be decontaminated by appropriate means	Yes	Yes	Yes

Requirements of the laboratory/animal facility	Containment Group		
	2	**3**	**4**
38. Any accidents or incidents shall be recorded and reported to the Safety Officer	Yes	Yes	Yes
39. On entering all clothing removed and clean clothes put on		Yes	Yes
40. On exiting all laboratory clothes removed, individual shall wash and transfer to clean side		Yes	
41. Individual shall shower prior to transfer to clean side			Yes
D) Handling of specimens			
42. Packaging requirements to be advised prior to submission	Yes	Yes	Yes
43. Incoming packages opened by trained staff in appropriately contained reception area	Yes	Yes	Yes
44. Movement of pathogens from an approved laboratory to another requires a licence	Yes	Yes	Yes
45. Standard Operating Procedures covering all areas must be available	Yes	Yes	Yes

1. Additional requirements for work in animal facilities

1. The animal facility should be easy to clean, with surfaces that are impervious to water and resistant to chemicals used in the area.

2. Personnel access to the work area should be restricted; appropriate security measures such as controlled electronic access may be necessary with higher risk agents.

3. Personal protective equipment such as coveralls, boots, disposable gloves, masks, safety glasses, face shields, and oro-nasal respirators, as appropriate, shall be worn in the animal facility and removed when leaving the animal facility.

4. The animal facility door should be closed when work is in progress and ventilation should be provided by extracting air from the room. (Where biosafety cabinets are used, care shall be taken to balance ventilation systems.)

5. Food (including chewing gum, candy, throat lozenges and cough drops) and/or drink shall not be stored or consumed in animal facilities.

6. Smoking and/or application of cosmetics shall not take place in the animal facility.

10. Used laboratory glassware and other materials shall be stored safely before disinfection. Materials for disposal shall be transported without spillage in strong containers. Waste material should be autoclaved, incinerated or otherwise made safe before disposal. Reusable material shall be decontaminated by appropriate means.

11. No infectious material shall be discarded down animal facility drains without appropriate waste treatment in place.

12. Any accidents or incidents shall be recorded and reported to the Safety Officer.

L. CONCLUSION

High standards of laboratory safety and containment that will ensure healthy working conditions for laboratory staff and protection of the environment must be of the greatest priority. They can only be achieved by careful study of the principles involved followed by practical application to premises, facilities, operating procedures and hygiene. Training of all laboratory personnel must be a high priority and no personnel should be allowed to work until appropriate training and competence has been demonstrated and documented. There is a large published literature on all aspects of the subject, and further reading is recommended (Beran & Steele, 1994; European Committee for Standardisation, 2000; Richmond, 1996–2002; Sewell, 1995; World Health Organization, 2004).

REFERENCES

ACHA P.N. & SZYFRES B. (2001). Zoonoses and Communicable Diseases Common to Man and Animals, Third Edition. Pan American Health Organisation, Scientific Publication. PAHO, Washington DC, USA.

ADVISORY COMMITTEE ON DANGEROUS PATHOGENS (1995). Categorisation of Biological Agents According to Hazard and Categories of Containment, Fourth Edition. Health and Safety Executive Books, P.O. Box 1999, Sudbury, Suffolk, CO10 6FS, UK. ISBN 0-7176-1038-1.

ADVISORY COMMITTEE ON DANGEROUS PATHOGENS (1998). Transmissible Spongiform Encephalopathy Agents: Safe Working and the Prevention of Infection. Spongiform Encephalopathy Advisory Committee. Her Majesty's Stationery Office, London, UK.

BARBEITO M.S., ABRAHAM G., BEST M., CAIRNS P., LANGEVIN P., STERRITT W.G., BARR D., MEULEPAS W., SANCHEZ-VIZCAINO J.M., SARAZA M., REQUENA E., COLLADO M., MANI P., BREEZE R., BRUNNER H., MEBUS C.A., MORGAN R.L., RUSK S., SIEGFRIED L.M. & THOMPSON L.H. (1995). Recommended biocontainment features for research and diagnostic facilities where animal pathogens are used. *Rev. sci. tech. Off. int. Epiz.*, **14**, 873–887.

BELL J.C., PALMER S.R. & PAYNE J.M. (1988). The Zoonoses. Edward Arnold, London, UK.

BERAN G.W. & STEELE J.H. (eds) (1994). Handbook of Zoonoses, Second Edition. Section A. (Bacterial, Rickettsial, Chlamydial and Mycotic Zoonoses) and Section B (Viral Zoonoses), CRC Press, USA.

CANADIAN FOOD INSPECTION AGENCY (1996). Containment Standards for Veterinary Facilities, Best M., ed. Canadian Food Inspection Agency, Ottawa, Ontario, Canada.

COLLINS C.H. (1990). Health hazards in microbiology. *In:* Handbook of Laboratory Health and Safety Measures, Pal S.B., ed. Kluwer Academic Publications, Dordrecht, The Netherlands, 159–188.

DEPARTMENT OF HEALTH AND HUMAN SERVICES (DHHS) PUBLIC HEALTH SERVICE, CENTRES FOR DISEASE CONTROL AND PREVENTION (CDC), AND NATIONAL INSTITUTES OF HEALTH (NIH) (2009). Biosafety in Microbiological and Biomedical Laboratories, Fifth Edition. HHS Publication No. (CDC) 21-1112. Revised December 2009. US Government Printing Office, Washington DC, USA. http://www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm

DEPARTMENT OF HEALTH AND HUMAN SERVICES (DHHS) PUBLIC HEALTH SERVICE, CENTRES FOR DISEASE CONTROL AND PREVENTION (CDC), AND NATIONAL INSTITUTES OF HEALTH (NIH) (2000). Primary Containment for Biohazards: Selection, Installation and Use of Biological Safety Cabinets, Second Edition. US Government Printing Office, Washington DC, USA.

EUROPEAN COMMITTEE FOR STANDARDISATION (2000). EN12469:2000; Performance Criteria for Microbiological Safety Cabinets. European Committee for Standardisation, Rue de Strassart 36, B-1040 Brussels, Belgium.

INTERNATIONAL ATOMIC ENERGY AGENCY (IAEA) (1994). International Basic Safety Standards for Protection against Ionizing Radiation and for the Safety of Radiation Sources, Interim Edition. Safety Series No. 115-I. IAEA, Vienna, Austria.

OFFICE OF BIOSAFETY, LABORATORY CENTRE FOR DISEASE CONTROL, HEALTH AND WELFARE CANADA (1996). Laboratory Biosafety Guidelines, Second Edition. Ministry of Supply and Services Canada, Cat. No. MR 21-2/1996.

RAYBURN S.R. (1990). The Foundations of Laboratory Safety (a Guide for the Biomedical Laboratory). Springer-Verlag, New York, USA.

RICHMOND Y. (ed) (1996–2002). Anthology of Biosafety: I–V. American Biological Safety Association. Mundelein, Illinois, USA.

SEWELL D.L. (1995). Laboratory associated infections and biosafety. *Clin. Microbiol. Rev.*, **8**, 389–405.

UNITED STATES DEPARTMENT OF AGRICULTURE (USDA) (2002). ARS Facilities Design Standards, Section 9 Biohazard Containment Design. http://www.afm.ars.usda.gov/ppweb/pdf/242-01m.pdf

WOOD M. & SMITH M.W. (EDS) (1999). Health and Safety in Laboratory Animal Facilities. Royal Society of Medicine Press, London, UK, pp. 249.

WORLD HEALTH ORGANIZATION (2004). Laboratory Biosafety Manual, Third Edition. WHO, Geneva, Switzerland.

*

* *

CHAPTER 1.1.4.

QUALITY MANAGEMENT IN VETERINARY TESTING LABORATORIES

SUMMARY

Valid laboratory results are essential for diagnosis, surveillance and trade. Such results are achieved by the use of good management practices, valid test and calibration methods, proper technique, quality control and quality assurance, all working together within a quality management system. Laboratory quality management includes technical, managerial and operational elements of testing and the interpretation of test results. A quality management system enables the laboratory to demonstrate both competency and an ability to generate consistent technically valid results that meet the needs of its customers. The need for mutual recognition of test results for international trade and the acceptance of international standards such as ISO/IEC[1] 17025:2005 General Requirements for the Competence of Testing and Calibration Laboratories (ISO/IEC, 2005) requires good laboratory quality management systems. The OIE has published a detailed standard on this subject (OIE, 2008). This chapter is not intended to reiterate the requirements of these two documents, nor has it been endorsed by accreditation bodies. Rather, it outlines the important issues and considerations a laboratory should address in the design and maintenance of its quality management system, whether or not it has been formally accredited.

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

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

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

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

i) The type of testing done;

ii) The purpose and requirements of the test results;

iii) The impact of a questionable or erroneous result;

iv) The tolerance level of risk and liability;

v) Customer needs (e.g. sensitivity and specificity of the test method, cost, turnaround time, strain/genotype characterisation);

vi) The role of the laboratory in legal work or in regulatory programmes;

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

viii) The business goals of the laboratory, including the need for any third party recognition and/or accreditation.

---

1 ISO/IEC - International Organization for Standardization/International Electrochemical Commission.

2. Standards, guides, and references

The laboratory should choose reputable and accepted standards and guides to assist in designing the quality management system. The OIE standard on this subject is a useful guideline (OIE, 2008). For laboratories seeking accreditation of testing, the use of ISO/IEC 17025 (ISO/IEC, 2005) or the OIE Standard (2008) will be essential. Further information on standards may be obtained from the national standards body of each country, from the International Laboratory Accreditation Cooperation (ILAC), and from accreditation bodies, e.g. the National Association of Testing Authorities (NATA), Australia, the United Kingdom Accreditation Service (UKAS), the American Association for Laboratory Accreditation (A2LA), etc. Technical and international organisations such as AOAC International (The Scientific Association Dedicated to Analytical Excellence; formerly the Association of Official Analytical Chemists) and the International Organization for Standardization (ISO) publish useful references, guides and standards that supplement the general requirements of ISO/IEC 17025. The ISO International Standard 9001 (ISO, 2008), is a certification standard for quality management systems and while it may be a useful supplement to a quality system, its requirements do not necessarily ensure or imply technical competence (in the areas listed Section 3 below). ISO 9001 is assessed by a certification body, which is accredited to undertake such assessments by the national accreditation body. When a laboratory meets the requirements of ISO 9001, the term *registration* or *certification* is used to indicate conformity, not *accreditation*.

3. Accreditation

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

i)   Has technically valid and validated test methods, procedures and specifications that are documented in accordance with the requirements of the applicable standard or guidelines;

ii)   Has appropriately qualified and trained personnel with a depth of technical knowledge commensurate with appropriate levels of authority;

iii)   Has appropriate equipment with planned maintenance/calibration schedules;

iv)   Has adequate facilities and environmental control;

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

vi)   Implements continual improvements in testing and quality management;

vii)   Can assess the need for and implement appropriate corrective or preventive actions;

viii)   Accurately assesses and controls uncertainty in testing;

ix)   Demonstrates proficiency in the test methods used (e.g. by participation in proficiency tests on a regular basis);

x)   Has demonstrated competence to generate technically valid results.

4. Selection of an accreditation body

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

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

The quality management system should cover all areas of activity affecting all testing that is done at the laboratory. While accredited laboratories are obliged to meet the requirements of the standard as detailed below, these principles are relevant to all testing laboratories.

Laboratories accredited to ISO/IEC 17025 have a specific list of those tests that are accredited, called the schedule of accreditation or the scope. If new testing methods are introduced these must be assessed and accredited before they can be added to the scope. The quality management system should ideally cover all areas of activity affecting all testing that is done at the laboratory. However, it is up to the laboratory to decide which tests are to be accredited and included in the scope. If an accredited laboratory also offers unaccredited tests, these must be clearly indicated as such on any reports that claim or make reference to accreditation. Factors that might affect the laboratory's choice of tests for scope of accreditation include:

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

ii)   A contractual requirement for accredited testing (e.g. for international trade, research projects);

iii)   The importance of the test and the impact of an incorrect result;

iv)   The cost of maintaining an accredited test;

v)   Availability of personnel, facilities and equipment;

vi)   Availability of reference standards (e.g. standardised reagents, internal quality control samples, reference cultures) and proficiency testing schemes;

vii)   The quality assurance necessary for materials, reagents and media;

viii)   The validation, technical complexity and reliability of the test method;

ix)   The potential for subcontracting of accredited tests;

6.   Quality Assurance, Quality Control and Proficiency testing

Quality Assurance (QA) is the systematic and planned process of ensuring that the service offered meets the stated requirements in all areas. The requirements may be internal or defined in an accreditation/certification standard. QA is process orientated and ensures the right things are being done in the right way.

Quality Control (QC) is the systematic and planned monitoring of output to ensure the minimum levels of quality have been met. For a testing laboratory, this is to ensure test processes are working correctly and results are within the expected parameters and limits. QC is test orientated and ensures the results are as expected.

Proficiency Testing (PT), sometimes referred to as External Quality Assurance or EQA, is the determination of a laboratory's performance by testing specimens of undisclosed content. Ideally, PT schemes should be run by an external independent provider. Participation in proficiency testing enables the laboratory to assess and demonstrate the reliability results by comparison with those from other participating laboratories.

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

Providers and operators of proficiency testing programmes should be accredited to ISO/IEC 17043:2010 – Conformity assessment – General requirements for proficiency testing (ISO/IEC, 2010). This replaces the ISO/IEC Guide 43–1:1997 Proficiency testing by interlaboratory comparisons on which the previous OIE Guidelines were based (OIE, 2008, Guide 4).

Proficiency testing material from accredited providers has been well characterised and any spare material, once the proficiency testing has been completed, can be useful to demonstrate staff competence or for test validation.

7.   Test methods

ISO/IEC 17025 requires the use of appropriate test methods and has requirements for their selection, development, and validation. The OIE Quality Standard and Guidelines for Veterinary Laboratories: Infectious Diseases (OIE, 2008) also provides requirements for selection and validation.

This *Terrestrial Manual* provides recommendations on the selection of test methods for trade, diagnostic and surveillance purposes in the chapters on specific diseases. In addition, a list of prescribed tests for international trade is provided. As stated in the introduction to this list, the prescribed tests that are listed are those that are required by the OIE *Terrestrial Animal Health Code.* These tests are considered to be adequately validated to give reliable results to qualify animals for international movement. Also listed are alternative tests that may be

suitable for use within a local setting, but that may have limited validation. The fact that a test is recommended does not necessarily mean that a laboratory is competent to perform it. The laboratory quality system should incorporate provision of evidence of competency.

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

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

This *Terrestrial Manual* provides more detailed and specific guidance on test selection, optimisation, standardisation, and validation in Chapter 1.1.4/5 Principles and methods of validation of diagnostic assays for infectious diseases. The following are key test method issues for those involved in the quality management of the laboratory.

a) Selection of the test method

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

i) International acceptance;

ii) Scientific acceptance;

iii) Appropriate or current technology;

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

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

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

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

viii) Test turnaround time;

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

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

xi) Safety factors;

xii) Customer expectations;

xiii) Throughput of test samples required;

xiv) Cost of test, per sample;

xv) Availability of reference standards, reference materials and proficiency testing schemes.

b) Optimisation and standardisation of the test method

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

i) Critical specifications for equipment and instruments;

ii) Critical specifications for reagents (e.g. chemicals, biologicals), reference standards, reference materials, and internal controls;

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

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

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

vi)   Criteria for non-subjective acceptance or rejection of a batch of test results;

vii)   Criteria for the interpretation and reporting of test results;

viii) A documented test method and reporting procedure for use by laboratory staff;

ix)   Evidence of technical competence for those who perform the test processes and interpret results.

c)   Validation of the test method

Validation evaluates the test for its fitness for a given use by establishing test performance characteristics, such as sensitivity, specificity, and isolation rate; and diagnostic parameters such as positive/negative cut-off, and titre of interest or significance. Validation should be done using an optimised, documented, and fixed procedure. The extent and depth of the validation process will depend on logistical and risk factors. It may involve any number of activities and amount of data, with subsequent data analysis using appropriate statistical methods. Validation activities might include:

i)   Field and/or epidemiological studies;

ii)   Repeat testing to establish the effect of variables such as operator, reagents, equipment;

iii)   Comparison with other, preferably standard, methods and with reference standards (if available);

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

v)   Reproduction of data from an accepted standard method, or from a reputable publication;

vi)   Experimental infection or disease outbreak studies;

vii)   Analysis of internal quality control data.

Validation is always a balance between cost, risk, and technical possibilities. There may be cases where quantities such as accuracy and precision can only be given in a simplified way. Criteria and procedures for the correlation of test results for diagnosis of disease status or for regulatory action must be developed. The criteria and procedures developed should account for screening methods, retesting and confirmatory testing.

Test validation is covered in Chapter 1.1.4/5 Principles and methods of validation of diagnostic assays for infectious diseases.

d)   Uncertainty of the test method

Measurement of Uncertainty (MU) is "a parameter associated with the result of a measurement that characterises the dispersion of values that could reasonably be attributed to the measure" (Eurachem, 2000). Uncertainty of measurement does not imply doubt about a result but rather increases confidence in its validity. It is not the equivalent to *error*, as it may be applied to all test results derived from a particular procedure.

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

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

The determination of MU is well established in *quantitative* measurement sciences (ANSI, 1997). It may be given as a numeric expression of reliability and is commonly shown as a stated range. Standard deviation (SD) and confidence interval (CI) are examples of the expression of MU, for example the Optical Density result of an ELISA expressed as ± n SD, where n is usually 1, 2 or 3. The Confidence Interval (usually 95%) gives an estimated range in which the result is likely to fall, calculated from a given set of test data.

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

performance of the method and making use of previous experience, validation data, internal control results etc.

Many technical organisations and accreditation bodies (e.g. AOAC International, ISO, NATA, A2LA, SCC, UKAS, Eurachem, CITAC) teach courses and/or provide guidance on MU for laboratories seeking accreditation.

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

i) Sampling;

ii) Contamination;

iii) Sample transport and storage conditions;

iv) Sample processing;

v) Reagent quality, preparation and storage;

vi) Type of reference material;

vii) Volumetric and weight manipulations;

viii) Environmental conditions;

ix) Equipment effects;

x) Analyst or operator bias;

xi) Biological variability;

xii) Unknown or random effects.

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

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

Additional information on the analysis of uncertainty may be found in the Eurachem Guides to Quantifying Uncertainty in Measurement (Eurachem, 2000) and Use of uncertainty information in compliance assessment (Eurachem, 2007).

e) **Implementation and use of the test method**

Training should be a planned and structured activity with steps to ensure adequate supervision is maintained while analysts are being trained. Analysts should be able to demonstrate proficiency in using the test method prior to producing reported results, and on an ongoing basis.

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

8. Strategic planning

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

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

ii)   Participation in local and international organisations;

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

iv)   Current awareness of publications, writing and reviewing publications about diagnostic methods;

v)   Training programmes, including visits to other laboratories;

vi)   Conducting research;

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

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

ix)   Planned, continual professional development and technical training;

x)   Management reviews;

xi)   Analysis of customer feedback;

xii) Root cause analysis of anomalies and implementation of corrective, preventive and improvement actions.

REFERENCES

ANSI (AMERICAN NATIONAL STANDARDS INSTITUTE) (1997). ANSI/NCSL[2] Z540-2-1997, US Guide to the Expression of Uncertainty in Measurement, First Edition. American National Standards Institute, www.ansi.org.

EURACHEM (2000). Eurachem/CITAC[3] Guide: Quantifying Uncertainty in Analytical Measurement, Second Edition. Eurachem Secretariat. www.eurachem.org.

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

ILAC (2007). G13:08/2007 Guidelines for the Requirements for the Competence of Providers of Proficiency Testing Schemes. International Laboratory Accreditation Cooperation (ILAC). www.ilac.org

ISO (2004) ISO/TS 21748. Guidance for the use of Repeatability, Reproducibility and Trueness Estimates in Measurement Uncertainty Estimation. International Organization for Standardisation (ISO), www.iso.org.

ISO (2008). ISO 9001:2008. Quality Management Systems – Requirements. International Organization for Standardization (ISO), www.iso.org.

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

ISO/IEC (2004a). ISO/IEC 17011:2004. Conformity Assessment – General requirements for accreditation bodies accrediting conformity assessment bodies. International Organization for Standardization (ISO), www.iso.org.

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

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

OIE (WORLD ORGANISATION FOR ANIMAL HEALTH) (2008). Standard for Management and Technical Requirements for Laboratories Conducting Tests for Infectious Animal Diseases. *In:* OIE Quality Standard and Guidelines for Veterinary Laboratories: Infectious Diseases, Second Edition. World Organisation for Animal Health (OIE: Office International des Epizooties), 12 rue de Prony, 75017 Paris, France. www.oie.int.

*
*   *

---

2   NCSL: The National Conference of Standards Laboratories.
3   CITAC: The Cooperation of International Traceability in Analytical Chemistry.

CHAPTER 2.4.3.

BOVINE BRUCELLOSIS

SUMMARY

Bovine brucellosis is usually caused by Brucella abortus*, less frequently by* B. melitensis*, and occasionally by* B. suis*. Infection is widespread globally. Several countries in Northern and Central Europe, Canada, Japan, Australia and New Zealand are believed to be free from the agent.*

Clinically, the disease is characterised by one or more of the following signs: abortion, retained placenta, orchitis, epididymitis and, rarely, arthritis, with excretion of the organisms in uterine discharges and in milk. Diagnosis depends on the isolation of Brucella *from abortion material, udder secretions or from tissues removed at post-mortem. Presumptive diagnosis can be made by assessing specific cell-mediated or serological responses to* Brucella *antigens.*

Brucella abortus, B. melitensis *and* B. suis *are highly pathogenic for humans, and all infected tissues, cultures and potentially contaminated materials must be handled under appropriate containment conditions.*

Identification of the agent: *Presumptive evidence of* Brucella *is provided by the demonstration, by modified acid-fast staining of organisms, of* Brucella *morphology in abortion material or vaginal discharge, especially if supported by serological tests. The polymerase chain reaction methods provide additional means of detection. Whenever possible,* Brucella *spp. should be isolated using plain or selective media by culture from uterine discharges, aborted fetuses, udder secretions or selected tissues, such as lymph nodes and male and female reproductive organs. Species and biovars should be identified by phage lysis, and by cultural, biochemical and serological criteria. Polymerase chain reaction (*PCR*) can provide both a complementary and biotyping method based on specific genomic sequences.*

Serological and allergic skin tests: *The buffered* Brucella *antigen tests, i.e. Rose Bengal test and buffered plate agglutination test, the complement fixation test, the enzyme-linked immunosorbent assay (*ELISA*) or the fluorescence polarisation assay, are suitable tests for screening herds and individual animals. However, no single serological test is appropriate in each and all epidemiological situations. Therefore, the reactivity of samples that are positive in screening tests should be assessed using an established confirmatory and/or complementary strategy. The indirect ELISA or milk ring test performed on bulk milk samples are effective for screening and monitoring dairy cattle for brucellosis, but the milk ring test is less reliable in large herds. Another immunological test is the brucellin skin test, which can be used as a screening or as a confirmatory herd test when positive serological reactors occur in the absence of obvious risk factors in unvaccinated herds.*

Requirements for vaccines and diagnostic biologicals: Brucella abortus *strain 19 remains the reference vaccine to which any other vaccines are compared. It should be prepared from US-derived seed cultures with adequate residual virulence and immunogenicity to protect mice against challenge with a virulent strain of* B. abortus*. Moreover each batch must conform to minimum standards for viability, smoothness, and designated CFU (*colony-forming units*) per dose.* Brucella abortus *strain RB51 vaccine was produced from a laboratory-derived rough mutant of smooth* B. abortus *strain 2308. It has become the official vaccine for prevention of brucellosis in cattle in some countries. Brucellin preparations for the intradermal test must be free of smooth lipopolysaccharide and must not produce nonspecific inflammatory reactions or interfere with serological tests. Diagnostic antigens must be prepared from smooth strains of* B. abortus*, strain 1119-3 or strain 99 and comply with minimum standards for purity, sensitivity and specificity.*

A. INTRODUCTION

Brucellosis in cattle is usually caused by biovars of *Brucella abortus*. In some countries, particularly in southern Europe and western Asia, where cattle are kept in close association with sheep or goats, infection can also be caused by *B. melitensis* (38, 87). Occasionally, *B. suis* may cause a chronic infection in the mammary gland of cattle, but it has not been reported to cause abortion or spread to other animals (24). The disease is usually asymptomatic in nonpregnant females. Following infection with *B. abortus* or *B. melitensis*, pregnant adult females develop a placentitis usually resulting in abortion between the fifth and ninth month of pregnancy. Even in the absence of abortion, profuse excretion of the organism occurs in the placenta, fetal fluids and vaginal discharges. The mammary gland and associated lymph nodes may also be infected, and organisms may be excreted in the milk. Subsequent pregnancies are usually carried to term, but uterine and mammary infection recurs, with reduced numbers of organisms in cyetic products and milk. In acute infections, the organism is present in most major body lymph nodes. Adult male cattle may develop orchitis and brucellosis may be a cause of infertility in both sexes. Hygromas, usually involving leg joints, are a common manifestation of brucellosis in some tropical countries and may be the only obvious indicator of infection; the hygroma fluid is often infected with *Brucella*.

Brucellosis has been reported in the one-humped camel (*Camelus dromedarius*) and in the two-humped camel (*C. bactrianus*), and in the South American camelids, llama (*Lama glama*), alpaca (*Lama pacos*), guanaco (*Lama guinicoe*), and vicuna (*Vicugne vicugne*) related to contact with large and small ruminants infected with *B. abortus* or *B. melitensis.* In addition, brucellosis has been observed in the domestic buffalo (*Bubalus bubalus*), American and European bison *(Bison bison, Bison bonasus)*, yak (*Bos grunniens),* elk/wapiti (*Cervus elaphus*) and also occurs in the African buffalo (*Syncerus caffer*) and various African antelope species. The clinical manifestations of brucellosis in these animals are similar to those in cattle.

The World Health Organization (WHO) laboratory biosafety manual classifies *Brucella* in Risk group III. Brucellosis is readily transmissible to humans, causing acute febrile illness – undulant fever – which may progress to a more chronic form and can also produce serious complications affecting the musculo–skeletal, cardiovascular, and central nervous systems. Precautions should be taken to prevent human infection. Infection is often due to occupational exposure and is essentially acquired by the oral, respiratory, or conjunctival routes, but ingestion of dairy products constitutes the main risk to the general public where the disease is endemic. There is an occupational risk to veterinarians and farmers who handle infected animals and aborted fetuses or placentas. Brucellosis is one of the most easily acquired laboratory infections, and strict safety precautions should be observed when handling cultures and heavily infected samples, such as products of abortion. Specific recommendations have been made for the biosafety precautions to be observed with *Brucella*-infected materials (for further details see refs 1, 39, 94 and Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiological laboratory and animal facilities). Laboratory manipulation of live cultures or contaminated material from infected animals is hazardous and must be done under containment level 3 or higher, as outlined in Chapter 1.1.2, to minimise occupational exposure. Where large-scale culture of *Brucella* is carried out (e.g. for antigen or vaccine production) then biosafety level 3 is essential.

Genetic and immunological evidence indicates that all members of the *Brucella* genus are closely related Nevertheless, based on relevant differences in host preference and epidemiology displayed by the major variants, as well as molecular evidence of genomic variation, the International Committee on Systematics of Prokaryotes, Subcommittee on the Taxonomy of *Brucella* took a clear position in 2005 on a return to pre-1986 *Brucella* taxonomic opinion; the consequences of this statement imply the re-approval of the six *Brucella* nomenspecies with recognised biovars. The classical names related to the six *Brucella* nomenspecies are validly published in the Approved Lists of Bacterial Names, 1980, and the designated type strains are attached to these validly published names: *Brucella abortus, B. melitensis, B. suis, B. neotomae, B. ovis* and *B. canis* (http://www.the-icsp.org/subcoms/Brucella.htm). The first three of these are subdivided into biovars based on cultural and serological properties (see Tables 1 and 2). Strains of *Brucella* have been isolated in the last decade from marine mammals that cannot be ascribed to any of the above-recognised species. Investigations are continuing to establish their correct position in the taxonomy of that genus and it is proposed that they could be classified into two new species, B. *ceti* and B. *pinnipedialis* (26). A new strain, named *Brucella microti,* was recently isolated from the common vole (*Microtus arvalis*) in Central Europe (76, 77). Finally, *Brucella* shows close genetic relatedness to some plant pathogens and symbionts of the genera *Agrobacterium* and *Rhizobium*, as well as, animal pathogens (*Bartonella*) and opportunistic or soil bacteria (*Ochrobactrum*).

Table 1. *Differential characteristics of species of the genus* Brucella

Species	Colony morphology[b]	Serum requirement	Lysis by phages[a] Tb RTD[c]	Lysis by phages[a] Tb 10⁴RTD	Lysis by phages[a] Wb RTD	Lysis by phages[a] Iz$_1$ RTD	Lysis by phages[a] R/C RTD	Oxidase	Urease activity	Preferred host
B. abortus	S	–[d]	+	+	+	+	–	+[e]	+[f]	Cattle and other Bovidae
B. suis	S	–	–	+	+[g]	+[g]	–	+	+[h]	Biovar 1: swine / Biovar 2: swine, hare / Biovar 3: swine / Biovar 4: reindeer / Biovar 5: wild rodents
B. melitensis	S	–	–	–	–[i]	+	–	+	+[j]	Sheep and goats
B. neotomae	S	–	–[k]	+	+	+	–	–	+[h]	Desert wood rat[l]
B. ovis	R	+	–	–	–	–	+	–	–	Rams
B. canis	R	–	–	–	–	–	+	+	+[h]	Dogs
B. ceti	S	+[m]		+[n]	+[o]	–	+	+	Cetaceans
B. pinnipedialis	S	+[m]		+[n]	+[o]	–	+	+	Pinnipeds
B. microti	S	–	–	+	+	+	–	+	+	Common vole

From refs 1, 39.

a   Phages: Tbilisi (Tb), Weybridge (Wb), Izatnagar1(Iz$_1$) and R/C
b   Normally occurring phase: S: smooth, R: rough
c   RTD: routine test dilution
d   *B. abortus* biovar 2 generally requires serum for growth on primary isolation
e   Some African isolates of *B. abortus* biovar 3 are negative
f   Intermediate rate, except strain 544 and some field strains that are negative
g   Some isolates of *B. suis* biovar 2 are not or partially lysed by phage Wb or Iz$_1$
h   Rapid rate
i   Some isolates are lysed by phage Wb
j   Slow rate, except some strains that are rapid
k   Minute plaques
l   *Neotoma lepida*
m   Some isolates are lysed by Tb
n   Most isolates are lysed by Wb
o   Most isolates are lysed by Iz

Table 2. *Differential characteristics of the biovars of* Brucella *species*

Species	Biovar	CO_2 requirement	H_2S production	Growth on dyes[a] Thionin	Growth on dyes[a] Basic fuchsin	Agglutination with monospecific sera A	Agglutination with monospecific sera M	Agglutination with monospecific sera R
B. melitensis	1	–	–	+	+	–	+	–
	2	–	–	+	+	+	–	–
	3	–	–	+	+	+	+	–
B. abortus	1	+[b]	+	–	+	+	–	–
	2	+[b]	+	–	–	+	–	–
	3	+[b]	+	+	+	+	–	–
	4	+[b]	+	–	+[c]	–	+	–
	5	–	–	+	+	–	+	–
	6	–	–	+	+	+	–	–
	9	+ or –	+	+	+	–	+	–
B. suis	1	–	+	+	–[d]	+	–	–
	2	–	–	+	–	+	–	–
	3	–	–	+	+	+	–	–
	4	–	–	+	–[e]	+	+	–
	5	–	–	–	–	–	+	–
B. neotomae	–	–	+	–[f]	–	+	–	–
B. ovis	–	+	–	+	–[e]	–	–	+
B. canis	–	–	–	+	–[e]	–	–	+
B. ceti	–	–	–	+	+	+	–[e]	–
B. pinnipedialis	–	+	–	+	+	+	–[e]	–
B. microti	–	–	–	+	+	–	+	–

From refs 1, 39.

a   Dye concentration in serum dextrose medium: 20 µg/ml
b   Usually positive on primary isolation
c   Some basic fuchsin-sensitive strains have been isolated
d   Some basic fuchsin-resistant strains have been isolated
e   Negative for most strains
f   Growth at a concentration of 10 µg/ml thionin

B. DIAGNOSTIC TECHNIQUES

All abortions in cattle in late gestation, starting from the fifth month, should be treated as suspected brucellosis and should be investigated. The clinical picture is not pathognomonic, although the herd history may be helpful. Unequivocal diagnosis of *Brucella* infections can be made only by the isolation and identification of *Brucella*, but in situations where bacteriological examination is not practicable, diagnosis must be based on serological methods. There is no single test by which a bacterium can be identified as *Brucella*. A combination of growth characteristics, serological, bacteriological and/or molecular methods is usually needed.

1. Identification of the agent (1, 17, 18, 39)

a) Staining methods

Brucella are coccobacilli or short rods measuring from 0.6 to 1.5 µm long and from 0.5 to 0.7 µm wide. They are usually arranged singly, and less frequently in pairs or small groups. The morphology of *Brucella* is fairly constant, except in old cultures where pleomorphic forms may be evident. *Brucella* are nonmotile. They do not form spores, and flagella, pili, or true capsules are not produced. *Brucella* are Gram negative and usually do not show bipolar staining. They are not truly acid-fast, but are resistant to decolorisation by weak acids and thus stain red by the Stamp's modification of the Ziehl–Neelsen's method. This is the usual procedure for the examination of smears of organs or biological fluids that have been previously fixed with heat or ethanol, and by this method, *Brucella* organisms stain red against a blue background. A fluorochrome or peroxidase-labelled antibody conjugate based technique could also be used (72). The presence of intracellular, weakly acid-fast organisms of *Brucella* morphology or immuno-specifically stained organisms is presumptive evidence of brucellosis. However, these methods have a low sensitivity in milk and dairy products where *Brucella* are often present in small numbers, and interpretation is frequently impeded by the presence of fat globules. Care must be taken as well in the interpretation of positive results in the Stamps's method because other organisms that cause abortions, e.g. *Chlamydophila abortus* (formerly *Chlamydia psittaci*) or *Coxiella burnetii,* are difficult to differentiate from *Brucella* organisms. The results, whether positive or negative, should be confirmed by culture.

DNA probes or polymerase chain reaction (PCR) methods can be used also to demonstrate the agent in various biological samples (9).

b) Culture

i) *Basal media*

Direct isolation and culture of *Brucella* are usually performed on solid media. This is generally the most satisfactory method as it enables the developing colonies to be isolated and recognised clearly. Such media also limit the establishment of non-smooth mutants and excessive development of contaminants. However, the use of liquid media may be recommended for voluminous samples or for enrichment purpose. A wide range of commercial dehydrated basal media is available, e.g. *Brucella* medium base, tryptose (or trypticase)–soy agar (TSA). The addition of 2–5% bovine or equine serum is necessary for the growth of strains such as *B. abortus* biovar 2, and many laboratories systematically add serum to basal media, such as blood agar base (Oxoid) or Columbia agar (BioMérieux), with excellent results. Other satisfactory media, such as serum–dextrose agar (SDA) or glycerol dextrose agar, can be used (1). SDA is usually preferred for observation of colonial morphology. A nonselective, biphasic medium, known as Castañeda's medium, is recommended for the isolation of *Brucella* from blood and other body fluids or milk, where enrichment culture is usually advised. Castañeda's medium is used because brucellae tend to dissociate in broth medium, and this interferes with biotyping by conventional bacteriological techniques.

ii) *Selective media*

All the basal media mentioned above can be used for the preparation of selective media. Appropriate antibiotics are added to suppress the growth of organisms other than *Brucella*. The most widely used selective medium is the Farrell's medium (25), which is prepared by the addition of six antibiotics to a basal medium. The following quantities are added to 1 litre of agar: polymyxin B sulphate (5000 units = 5 mg); bacitracin (25,000 units = 25 mg); natamycin (50 mg); nalidixic acid (5 mg); nystatin (100,000 units); vancomycin (20 mg).

A freeze-dried antibiotic supplement is available commercially (Oxoid). However, nalidixic acid and bacitracin, at the concentration used in Farrell's medium, have inhibitory effects on some *B. abortus* and *B. melitensis* strains (49). Therefore the sensitivity of culture increases significantly by the simultaneous use of both Farrell's and the modified Thayer–Martin medium. Briefly, the modified Thayer–Martin's medium can be prepared with GC medium base (38 g/litre; Biolife Laboratories, Milan, Italy) supplemented with haemoglobin (10 g/litre; Difco) and colistin methanesulphonate (7.5 mg/litre), vancomycin (3 mg/litre), nitrofurantoin (10 mg/litre), nystatin (100,000 International Units [IU]/litre =

17.7 mg) and amphotericin B (2.5 mg/litre) (all products from Sigma Chemical, St Louis, United States of America [USA]) (49). Contrary to several biovars of *B. abortus*, growth of *B melitensis* is not dependent on an atmosphere of 5–10% CO_2 (Table 2).

As the number of *Brucella* organisms is likely to be lower in milk, colostrum and some tissue samples than in abortion material, enrichment is advisable. In the case of milk, results are also improved by centrifugation and culture from the cream and the pellet, but strict safety measures should be implemented in this case to avoid aerosols. Enrichment can be carried out in liquid medium consisting of serum–dextrose broth, tryptose broth (or trypticase)–soy broth (TSA) or *Brucella* broth supplemented with an antibiotic mixture of at least amphotericin B (1 μg/ml), and vancomycin (20 μg/ml) (all final concentrations). The enrichment medium should be incubated at 37°C in air supplemented with 5–10% (v/v) CO_2 for up to 6 weeks, with weekly subcultures on to solid selective medium. If preferred, a biphasic system of solid and liquid selective medium in the same bottle (Castañeda's method) may be used to minimise subculture. A selective biphasic medium composed of the basal Castañeda's medium with the addition of the following antibiotics to the liquid phase, is sometimes recommended for isolation of *Brucella* in milk (quantities are per litre of medium): polymyxin B (sulphate) (6000 units = 6 mg); bacitracin (25,000 units = 25 mg); natamycin (50 mg); nalidixic acid (5 mg); amphotericin B (1 mg); vancomycin (20 mg); D-cycloserine (100 mg).

All culture media should be subject to quality control and should support the growth of *Brucella* strains from small inocula or fastidious strains, such as *B. abortus* biovar 2.

On suitable solid media, *Brucella* colonies can be visible after a 2–3-day incubation period. After 4 days' incubation, *Brucella* colonies are round, 1–2 mm in diameter, with smooth margins. They are translucent and a pale honey colour when plates are viewed in the daylight through a transparent medium. When viewed from above, colonies appear convex and pearly white. Later, colonies become larger and slightly darker.

Smooth (S) *Brucella* cultures have a tendency to undergo variation during growth, especially with subcultures, and to dissociate to rough (R) forms. Colonies are then much less transparent, have a more granular, dull surface, and range in colour from matt white to brown in reflected or transmitted light. Checking for dissociation is easily tested by crystal violet staining: rough colonies stain red/violet and smooth colonies do not uptake dye or stain pale yellow. If the colonies are smooth, they should be checked against antiserum to smooth *B. abortus,* or preferably against anti-A and -M monospecific sera. In the case of non-smooth colonies, isolates should be checked with antiserum to *Brucella* R antigen. Changes in the colonial morphology are generally associated with changes in virulence, serological properties and/or phage sensitivity. Typical colonial morphology and positive agglutination with a *Brucella* antiserum provide presumptive identification of the isolate as *Brucella*. Subsequent full identification is best performed by a reference laboratory.

iii) *Collection and culture of samples*

For the diagnosis of animal brucellosis by cultural examination, the choice of samples usually depends on the clinical signs observed. The most valuable samples include aborted fetuses (stomach contents, spleen and lung), fetal membranes, vaginal secretions (swabs), milk, semen and arthritis or hygroma fluids. From animal carcasses, the preferred tissues for culture are those of the reticulo-endothelial system (i.e. head, mammary and genital lymph nodes and spleen), the late pregnant or early post-parturient uterus, and the udder. Growth normally appears after 3–4 days, but cultures should not be discarded as negative until 8–10 days have elapsed.

Tissues: Samples are removed aseptically with sterile instruments. The tissue samples are prepared by removal of extraneous material (e.g. fat), cut into small pieces, and macerated using a 'Stomacher' or tissue grinder with a small amount of sterile phosphate buffered saline (PBS), before being inoculated on to solid media.

Vaginal discharge: A vaginal swab taken after abortion or parturition is an excellent source for the recovery of *Brucella* and far less risky for the personnel than abortion material. The swab is then streaked on to solid media.

Milk: Samples of milk must be collected cleanly after washing and drying the whole udder and disinfecting the teats. It is essential that samples should contain milk from all quarters, and 10–20 ml of milk should be taken from each teat. The first streams are discarded and the sample is milked directly into a sterile vessel. Care must be taken to avoid contact between the milk and the milker's hands. The milk is centrifuged in conditions that avoid the risk of aerosol contamination to personnel, and the cream and deposit are spread on solid selective medium, either separately or mixed. If brucellae are present in bulk milk samples, their numbers are usually low, and isolation from such samples is very unlikely.

Dairy products: Dairy products, such as cheeses, should be cultured on the media described above. As these materials are likely to contain small numbers of organisms, enrichment culture is advised. Samples need to be carefully homogenised before culture, after they have been ground in a tissue grinder or macerated and pounded in a 'Stomacher' or an electric blender with an appropriate volume of sterile PBS. Superficial strata (rind and underlying parts) and the core of the product should be

cultured. As brucellae grow, survive or disappear quite rapidly, their distribution throughout the different parts of the product varies according to the local physico-chemical conditions linked to specific process technologies.

All samples should be cooled immediately after they are taken, and transported to the laboratory in the most rapid way. On arrival at the laboratory, milk and tissue samples should be frozen if they are not to be cultured immediately.

Use of laboratory animals should be avoided unless absolutely necessary, but may sometimes provide the only means of detecting the presence of *Brucella,* especially when samples have been shown to be heavily contaminated or likely to contain a low number of *Brucella* organisms. Animal inoculation may be either subcutaneously or through abraded skin in guinea-pigs or, preferably, intravenously or intraperitoneally in mice. This work must be carried out under appropriate biosafety conditions as outlined in Chapter 1.1.2. The spleens of mice are cultured 7 days after inoculation and, for guinea-pigs, a serum sample is subjected to specific tests 3 and 6 weeks after inoculation, then the spleens are cultured.

c) Identification and typing

Any colonies of *Brucella* morphology should be checked using a Gram-stained (or a Stamp-stained) smear. As the serological properties, dyes and phage sensitivity are usually altered in the non-smooth phases, attention to the colonial morphology is essential in the typing tests described below. The recommended methods for observing colonial morphology are Henry's method by obliquely reflected light, the acriflavine test described by Braun & Bonestell, or White & Wilson's crystal violet method of staining colonies (1).

Identification of *Brucella* organisms can be carried out by a combination of the following tests: organism morphology after Gram or Stamp's staining, colonial morphology, growth characteristics, urease, oxidase and catalase tests, and the slide agglutination test with an anti-*Brucella* polyclonal serum. Species and biovar identification requires elaborate tests (such as phage lysis and agglutination with anti-A, -M or -R monospecific sera), the performance of which is left to reference laboratories with expertise in these methods. The simultaneous use of several phages e.g. Tbilissi (Tb), Weybridge (Wb), Izatnagar (Iz) and R/C provides a phage-typing system that, in experienced hands, allows a practical identification of smooth and rough species of *Brucella.* However, several characteristics, for example added CO_2 requirement for growth, production of H_2S (detected by lead acetate papers), and growth in the presence of basic fuchsin and thionin at final concentrations of 20 µg/ml, are revealed by routine tests that can be performed in moderately equipped nonspecialised laboratories (see Tables 1 and 2).

When sending *Brucella* strains to a reference laboratory for typing, it is essential that smooth colonies be selected. Cultures should be lyophilised and sealed in ampoules packed in screw-capped canisters or subcultured on to appropriate nutrient agar slopes contained in screw-capped bottles. The strains could also be sent suspended in transport media (e.g. Amies), but this could provide an opportunity for the establishment of rough mutants.

i)   *Brucella* organisms are among the most dangerous bacteria with which to work in terms of the risk of producing laboratory-acquired infections. For transporting *Brucella* cultures, the caps of the bottles or canisters should be screwed tightly down and sealed with PVC tapes. Bottles should be wrapped in absorbent paper or cotton wool, sealed in polyethylene bags and packed into a rigid container in accordance with the requirements of the International Air Transport Association (IATA) for shipping dangerous goods (36). These regulations are summarised in Chapter 1.1.1 Collection and shipment of diagnostic specimens, and they must be followed. As *Brucella* cultures are infectious agents, they are designated UN2814 and a Declaration of Dangerous Goods must be completed. There are also restrictions on submitting samples from suspected cases of brucellosis and the IATA regulations should be reviewed before sending samples (36). Other international and national guidelines should also be followed (95).

ii) Before dispatching cultures or diagnostic samples for culture, the receiving laboratory should be contacted to determine if a special permit is needed and if the laboratory has the capability to do the testing requested. If samples are to be sent across national boundaries, an import licence will probably be needed and should be obtained before the samples are dispatched (Chapter 1.1.2).

d) Nucleic acid recognition methods

The PCR, including the real-time format, provides an additional means of detection and identification of *Brucella* sp. (9, 11–13, 29, 35, 65). Despite the high degree of DNA homology within the genus *Brucella*, several molecular methods, including PCR, PCR restriction fragment length polymorphism (RFLP) and Southern blot, have been developed that allow, to a certain extent, differentiation between *Brucella* species and some of their biovars (for a review see refs 9 and 51). Pulse-field gel electrophoresis has been developed that allows the differentiation of several *Brucella* species (37, 50). *Brucella* biotyping and

distinguishing vaccine strains by PCR can be accomplished satisfactorily but there has been limited validation of the PCR for primary diagnosis.

The first species-specific multiplex PCR assay for the differentiation of *Brucella* was described by Bricker & Halling (12). The assay, named AMOS-PCR, was based on the polymorphism arising from species-specific localisation of the insertion sequence IS711 in the *Brucella* chromosome, and comprised five oligonucleotide primers that can identify without differentiating *B. abortus*, biovars 1, 2 and 4 but could not identify *B. abortus* biovars 3, 5, 6 and 9. Modifications to the assay have been introduced over time to improve performance, and additional strain-specific primers were incorporated for identification of the *B. abortus* vaccine strains, and other biovars and species (11, 13, 22, 23, 65).

A new multiplex PCR assay (Bruce-ladder) has been proposed for rapid and simple one-step identification of *Brucella* (29). The major advantage of this assay over previously described PCRs is that it can identify and differentiate in a single step most *Brucella* species as well as the vaccine strains *B. abortus* S19, *B. abortus* RB51 and *B melitensis* Rev.1. In contrast to other PCRs, Bruce-ladder is able to detect also DNA from *B. neotomae, B pinnipedialis* and *B ceti*. In addition, *B abortus* biovars 3, 5, 6, 7, 9, and *B. suis* biovars 2, 3, 4, 5 can be identified by this new multiplex PCR. The only minor inconvenience of the Bruce-ladder is that some *B canis* strains can be identified erroneously as *B suis* (46). Further, this assay cannot positively identify the new *B. microti* species.

- **Test procedure (Bruce-ladder multiplex PCR)**

i) *Brucella* DNA preparation

    Prepare bacteria from agar plates: with a sterile inoculating loop, transfer bacteria from one colony to 200 µl of saline. Extract the bacterial DNA by boiling for 10 minutes and, after centrifugation (12,000 ***g*** for 20 seconds), use 1.0 µl of the supernatant as a DNA template for PCR amplification (between 0.1 and 0.05 µg/µl of DNA, approximately).

ii) *Bruce*-ladder PCR mix preparation (per one reaction, final volume of 25 µl)

Reagents	Final concentration	Volume
PCR buffer 10×	1×	2.5 µl
dNTPs (2 mM)	400 µM each one	5.0 µl
Mg^{2+} (50 mM)	3.0 mM	1.5 µl
Bruce-ladder eight pair primer cocktail (12.5 µM)	6.25 pmol each one	7.6 µl
H$_2$O (PCR-grade)	–	7.1 µl
DNA polymerase*	1.5 U	0.3 µl

    *As this assay is a multiplex PCR with eight pairs of primers in the same tube reaction, best results are obtained when high quality DNA polymerase is used (for instance, Immolase DNA polymerase [Bioline], Titanium *Taq* DNA polymerase [Clontech], or PFU DNA polymerase [Biotools B&M Labs.]).**NOTE**: include always a negative control without DNA and a positive control with *B. suis* DNA

    Add 1.0 µl of template DNA

iii) Amplification by PCR

    Initial denaturation at 95°C for 7 minutes

    35 seconds of template denaturation at 95°C

    45 seconds of primer annealing at 64°C   for a total of 25 cycles

    3 minutes of primer extension at 72°C

    Final extension at 72°C for 6 minutes

iv) Detection of amplified product and interpretation of results

    Analyse the PCR products (7 µl) by electrophoresis (120 V for 1 hour) in a 1.5% agarose gel in TBE buffer (89 mM Tris/HCl, 89 mM boric acid, 2.0 mM ethylene diamino tetra-acetic acid [EDTA], pH 8.0). Use 1 kb plus DNA ladder as a molecular size marker. Visualise bands with UV light after staining with ethidium bromide. For interpretation of the results see reference 29.

Table 3. Oligonucleotides used in the Bruce-ladder multiplex PCR assay

Primer[a]	Sequence (5'–3')	Amplicon six (bp)	DNA targets	Source of genetic differences
BMEI0998f	ATC-CTA-TTG-CCC-CGA-TAA-GG	1682	Glycosyltransferase, gene *wboA*	IS*711* insertion in BMEI0998 in *B. abortus* RB51, and deletion of 15,079 bp in BMEI0993-BMEI1012 in *B. ovis*
BMEI0997r	GCT-TCG-CAT-TTT-CAC-TGT-AGC			
BMEI0535f	GCG-CAT-TCT-TCG-GTT-ATG-AA	450 (1320[b])	Immunodominant antigen, gene *bp26*	IS*711* insertion in BMEI0535-BMEI0536 in *Brucella* strains isolated from marine mammals
BMEI0536r	CGC-AGG-CGA-AAA-CAG-CTA-TAA			
BMEII0843f	TTT-ACA-CAG-GCA-ATC-CAG-CA	1071	Outer membrane protein, gene *omp31*	deletion of 25,061 bp in BMEII826-BMEII0850 in *B. abortus*
BMEII0844r	GCG-TCC-AGT-TGT-TGT-TGA-TG			
BMEI1436f	ACG-CAG-ACG-ACC-TTC-GGT-AT	794	Polysaccharide deacetylase	deletion of 976 bp in BMEI1435 in *B. canis*
BMEI1435r	TTT-ATC-CAT-CGC-CCT-GTC-AC			
BMEII0428f	GCC-GCT-ATT-ATG-TGG-ACT-GG	587	Erythritol catabolism, gene *eryC* (D-erythrulose-1-phosphate dehydrogenase)	deletion of 702 bp in BMEII0427-BMEII0428 in *B. abortus* S19
BMEII0428r	AAT-GAC-TTC-ACG-GTC-GTT-CG			
BR0953f	GGA-ACA-CTA-CGC-CAC-CTT-GT	272	ABC transporter binding protein	deletion of 2653 bp in BR0951-BR0955 in *B. melitensis* and *B. abortus*
BR0953r	GAT-GGA-GCA-AAC-GCT-GAA-G			
BMEI0752f	CAG-GCA-AAC-CCT-CAG-AAG-C	218	Ribosomal protein S12, gene *rpsL*	point mutation in BMEI0752 in *B. melitensis* Rev.1
BMEI0752r	GAT-GTG-GTA-ACG-CAC-ACC-AA			
BMEII0987f	CGC-AGA-CAG-TGA-CCA-TCA-AA	152	Transcriptional regulator, CRP family	deletion of 2,203 bp in BMEII0986-BMEII0988 in *B. neotomae*
BMEII0987r	GTA-TTC-AGC-CCC-CGT-TAC-CT			

[a]Designations are based on the *B. melitensis* (BME) or *B. suis* (BR) genome sequences. f: forward; r: reverse.
[b]Due to a DNA insertion in the *bp26* gene, the amplicon size in *Brucella* strains isolated from marine mammals is 1320 bp

Other tests such as as *omp*25, 2a and 2b PCR/RFLP (14, 15) are available and may be used to identify *Brucella* species.

Alternative approaches allowing identification of all *Brucella* species based on single nucleotide polymorphism (SNP) discrimination by either primer extension or real-time PCR have recently been described (32, 79). These tests are rapid, simple and unambiguous and, being based on a robust phylogenetic analysis, overcome some problems seen with Bruce-ladder, such as the misidentification of some *B. canis* isolates.

A number of other methods have recently been described that can add useful epidemiological information. These include a multilocus sequencing scheme (92) and several typing schemes based on the use of multiple locus variable number of tandem repeats analysis (MLVA) (10, 11, 42, 93). Depending on the

particular markers chosen, these methods allow isolates to be differentiated to the species level or to be further subdivided potentially providing valuable epidemiological information at the subspecies level.

e) Identification of vaccine strains

Identification of the vaccine strains *B. abortus* S19, *B. abortus* RB51 and *B. melitensis* strain Rev.1, depends on further tests.

Brucella abortus S19 has the normal properties of a biovar 1 strain of *B. abortus*, but does not require CO_2 for growth, does not grow in the presence of benzylpenicillin (3 µg/ml = 5 IU/ml), thionin blue (2 µg/ml), and i-erythritol (1 mg/ml) (all final concentrations), and presents a high L-glutamate use (1). In some cases strain 19 will grow in the presence of i-erythritol, but does not use it.

Brucella melitensis strain Rev.1 has the normal properties of a biovar 1 strain of *B. melitensis*, but develops smaller colonies on agar media, does not grow in the presence of basic fuchsin, thionin (20 µg/ml) or benzylpenicillin (3 µg/ml) (final concentrations), but does grow in the presence of streptomycin at 2.5 or 5 µg/ml (5 IU/ml) (1, 17, 18, 21).

Brucella abortus strain RB51 is identified by the following characteristics: rough morphology and growth in the presence of rifampicin (250 µg per ml of media).

Vaccine strains S19, Rev.1 and RB51 may also be identified using specific PCRs (13, 29, 75, 86, 88).

2. Serological tests

No single serological test is appropriate in all epidemiological situations; all have limitations especially when it comes to screening individual animals (31, 64). Consideration should be given to all factors that impact on the relevance of the test method and test results to a specific diagnostic interpretation or application. In epidemiological units where vaccination with smooth *Brucella* is practised, false-positive reactions may be expected among the vaccinated animals because of antibodies cross-reacting with wild strain infection. For the purposes of this chapter, the serological methods described represent standardised and validated methods with suitable performance characteristics to be designated as either prescribed or alternative tests for international trade. This does not preclude the use of modified or similar test methods or the use of different biological reagents. However, the methods and reagents described in this chapter represent a standard of comparison with respect to expected diagnostic performance.

It should be stressed that the serum agglutination test (SAT) is generally regarded as being unsatisfactory for the purposes of international trade. The complement fixation test (CFT) is diagnostically more specific than the SAT, and also has a standardised system of unitage. The diagnostic performance characteristics of some enzyme-linked immunosorbent assays (ELISAs) and the fluorescence polarisation assay (FPA) are comparable with or better than that of the CFT, and as they are technically simpler to perform and more robust, their use may be preferred (60, 97). The performances of several of these tests have been compared.

For the control of brucellosis at the national or local level, the buffered *Brucella* antigen tests (BBATs), i.e. the Rose Bengal test (RBT) and the buffered plate agglutination test (BPAT), as well as the ELISA and the FPA, are suitable screening tests. Positive reactions should be retested using a suitable confirmatory and/or complementary strategy.

In other species, for example, buffaloes (*Bubalus bubalus*), American and European bison (*Bison bison, Bison bonasus*), yak (*Bos grunniens*), elk/wapiti (*Cervus elaphus*), and camels (*Camelus bactrianus* and *C. dromedarius*), and South American camelids, *Brucella* sp. infection follows a course similar to that in cattle. The same serological procedures may be used for these animals (56), but each test should be validated in the animal species under study (27, 28).

• Reference sera

The OIE reference standards are those against which all other standards are compared and calibrated. These reference standards are all available to national reference laboratories and should be used to establish secondary or national standards against which working standards can be prepared and used in the diagnostic laboratory for daily routine use.

These sera have been developed and designated by the OIE as International Standard Sera[1]. The use of these promotes international harmonisation of diagnostic testing and antigen standardisation (97):

---

1   Obtainable from the OIE Reference Laboratory for Brucellosis at Veterinary Laboratories Agency (VLA) Weybridge, New Haw, Addlestone, Surrey KT15 3NB, United Kingdom.

- For RBT and CFT, the OIE International Standard Serum (OIEISS, previously the WHO Second International anti-*Brucella abortus* Serum) is used. This serum is of bovine origin and contains 1000 IU and ICFTU (international complement fixation test units).

- In addition, three OIE ELISA Standard Sera are available for use. These are also of bovine origin and consist of a strong positive (OIEELISA$_{SP}$SS), a weak positive (OIEELISA$_{WP}$SS) and a negative (OIEELISA$_N$SS) standard. Conditions for standardising FPA with these Standards need to be reviewed.

- **Production of cells**

  Brucella abortus strain 99 (Weybridge) (S99) (see footnote 1 for address) or *B. abortus* strain 1119-3 (USDA) (S1119-3)[2] should always be used for diagnostic antigen production. It should be emphasised that antigen made with one of these *B. abortus* strains is also used to test for *B. melitensis* or *B. suis* infection. The strains must be completely smooth and should not autoagglutinate in saline and 0.1% (w/v) acriflavine. They must be pure cultures and conform to the characteristics of CO_2-independent strains of *B. abortus* biovar 1. The original seed cultures should be propagated to produce a seed lot that must conform to the properties of these strains, and should be preserved by lyophilisation or by freezing in liquid nitrogen.

  For antigen production, the seed culture is used to inoculate a number of potato-infusion agar slopes that are then incubated at 37°C for 48 hours. SDA and TSA, to which 5% equine or newborn calf serum and/or 0.1% yeast extract may be added, are satisfactory solid media provided a suitable seed is used as recommended above. The growth is checked for purity, resuspended in sterile PBS, pH 6.4, and used to seed layers of potato-infusion agar or glycerol–dextrose agar in Roux flasks. These are then incubated at 37°C for 72 hours with the inoculated surface facing down. Each flask is checked for purity by Gram staining samples of the growth, and the organisms are harvested by adding 50–60 ml of phenol saline (0.5% phenol in 0.85% sodium chloride solution) to each flask. The flasks are gently agitated, the suspension is decanted, and the organisms are killed by heating at 80°C for 90 minutes. Following a viability check, the antigen is stored at 4°C.

  Alternatively, the cells may be produced by batch or continuous culture in a fermenter (34), using a liquid medium containing (per litre of distilled water) D-glucose (30 g), a high-grade peptone (30 g), yeast extract (Difco) (10 g), sodium dihydrogen phosphate (9 g) and disodium hydrogen phosphate (3.3 g). The initial pH is 6.6, but this tends to rise to pH 7.2–7.4 during the growth cycle. Care should be taken to check batches of peptone and yeast extract for capacity to produce good growth without formation of abnormal or dissociated cells. Vigorous aeration and stirring is required during growth, and adjustment to pH 7.2–7.4 by the addition of sterile 0.1 M HCl may be necessary. The seed inoculum is prepared as described above. The culture is incubated at 37°C for 48 hours. Continuous culture runs can be operated for much longer periods, but more skill is required to maintain them. In-process checks should be made on the growth from either solid or liquid medium to ensure purity, an adequate viable count and freedom from dissociation to rough forms. Cells for use in the preparation of all antigens should be checked for purity and smoothness at the harvesting stage.

  The culture is harvested by centrifugation to deposit the organisms, which are resuspended in phenol saline. The organisms are killed by heating at 80°C for 90 minutes and are stored at 4°C. They must form stable suspensions in physiological saline solutions and show no evidence of autoagglutination. A viability check must be performed on the suspensions and no growth must be evident after 10 days' incubation at 37°C. The packed cell volume (PCV) of the killed suspensions can be determined by centrifuging 1 ml volumes in Wintrobe tubes at 3000 **g** for 75 minutes.

a) **Buffered *Brucella* antigen tests (prescribed tests for international trade)**

- **Rose Bengal test**

  This test is a simple spot agglutination test using antigen stained with Rose Bengal and buffered to a low pH, usually 3.65 ± 0.05 (52).

  - **Antigen production**

  Antigen for the RBT is prepared by depositing killed *B. abortus* S99 or S1119-3 cells by centrifugation at 23,000 **g** for 10 minutes at 4°C, and uniformly resuspending in sterile phenol saline (0.5%) at the rate of 1 g to 22.5 ml. (Note: if sodium carboxymethyl cellulose is used as the sedimenting agent during preparation of the cell concentrate, insoluble residues must be removed by filtering the suspension through an AMF-CUNO Zeta-plus prefilter [Type CPR 01A] before staining.) To every 35 ml of this suspension, 1 ml of 1% (w/v) Rose Bengal (CI No. 45440) in sterile distilled water is added, and the mixture is stirred for 2 hours at room temperature. The mixture is filtered through sterile cotton wool, and centrifuged at 10,000 **g** to deposit the stained cells, which are then uniformly resuspended at the rate of 1 g cells to 7 ml of diluent (21.1 g of

---

2 Obtainable from the United States Department of Agriculture (USDA), National Veterinary Services Laboratories (NVSL), 1800 Dayton Road, Ames, Iowa 50010, United States of America.

sodium hydroxide dissolved in 353 ml of sterile phenol saline, followed by 95 ml of lactic acid, and adjusted to 1056 ml with sterile phenol saline). The colour of this suspension should be an intense pink and the supernatant of a centrifuged sample should be free of stain; the pH should be 3.65 ± 0.05. After filtration through cotton wool, the suspension is filtered twice through a Sartorius No. 13430 glass fibre prefilter, adjusted to a PCV of approximately 8%, pending final standardisation against serum calibrated against the OIEISS, and stored at 4°C in the dark. The antigen should be stored as recommended by the manufacturer but usually should not be frozen.

When used in the standard test procedure, the RBT antigen should give a clearly positive reaction with 1/45 dilution, but not 1/55 dilution, of the OIEISS diluted in 0.5% phenol saline or normal saline. It may also be advisable to compare the reactivity of new and previously standardised batches of antigen using a panel of defined sera.

- **Test procedure**

i)   Bring the serum samples and antigen to room temperature (22 ± 4°C); only sufficient antigen for the day's tests should be removed from the refrigerator.

ii)   Place 25–30 µl of each serum sample on a white tile, enamel or plastic plate, or in a WHO haemagglutination plate.

iii)   Shake the antigen bottle well, but gently, and place an equal volume of antigen near each serum spot.

iv)   Immediately after the last drop of antigen has been added to the plate, mix the serum and antigen thoroughly (using a clean glass or plastic rod for each test) to produce a circular or oval zone approximately 2 cm in diameter.

v)   The mixture is agitated gently for 4 minutes at ambient temperature on a rocker or three-directional agitator (if the reaction zone is oval or round, respectively).

vi)   Read for agglutination immediately after the 4-minute period is completed. Any visible reaction is considered to be positive. A control serum that gives a minimum positive reaction should be tested before each day's tests are begun to verify the sensitivity of test conditions.

The RBT is very sensitive. However, like all other serological tests, it could sometimes give a positive result because of S19 vaccination or of false-positive serological reactions (FPSR). Therefore positive reactions should be investigated using suitable confirmatory and/or complementary strategies (including the performance of other tests and epidemiological investigation). False-negative reactions occur rarely, mostly due to prozoning and can sometimes be detected by diluting the serum sample or retesting after 4–6 weeks. Nevertheless RBT appears to be adequate as a screening test for detecting infected herds or to guarantee the absence of infection in brucellosis-free herds.

- **Buffered plate agglutination test**

  - **Antigen production**

Antigen for the BPAT is prepared from *B. abortus* S1119-3 according to the procedure described by Angus & Barton (2).

Two staining solutions are required: brilliant green (2 g/100 ml) and crystal violet (1 g/100 ml) both certified stains dissolved in distilled water. Once prepared, the two solutions should be stored separately for a period of 24 hours, and then mixed together in equal volumes in a dark bottle and stored in a refrigerator for a period of not less than 6 months before use. The mixed stain may only be used between 6 and 12 months after initial preparation.

Buffered diluent is prepared by slowly dissolving sodium hydroxide (150 g) in 3–4 litres of sterile phenol saline. Lactic acid (675 ml) is added to this solution, and the final volume is adjusted to 6 litres by adding sterile phenol saline. The pH of the solution should be between 3.63 and 3.67.

Brucella abortus S1119-3 packed cells are diluted to a concentration of 250 g/litre in phenol saline; 6 ml of stain is added per litre of cell suspension, and the mixture is shaken thoroughly before being filtered through sterile absorbent cotton. The cells are centrifuged at 10,000 *g* at 4°C, and the packed cells are then resuspended at a concentration of 50 g/100 ml in buffered diluent (as described above). This mixture is shaken thoroughly for 2 hours, and is then further diluted by the addition of 300 ml of buffered diluent per 100 ml of suspended cells (i.e. final concentration of 50 g packed cells/400 ml buffered diluent). The mixture is stirred at room temperature for 20–24 hours before the cell concentration is adjusted to 11% (w/v) in buffered diluent. This suspension is stirred overnight before testing. Pending final quality control tests, the antigen is stored at 4°C until required for use. The antigen has a shelf life of 1 year and should not be frozen.

The pH of the buffered plate antigen should be 3.70 ± 0.03 and the pH of a serum:antigen mixture at a ratio of 8:3 should be 4.02 ± 0.04. The 11% stained-cell suspension should appear blue-green. Each batch of buffered plate antigen should be checked by testing at least 10 weakly reactive sera and comparing the results with one or more previous batches of antigen. If possible, the antigen batches should be compared with the standard antigen prepared by the NVSL, USDA (see footnote 2 for address). There is, however, no international standardisation procedure established for use with the OIEISS.

- **Test procedure**

i)   Bring the serum samples and antigen to room temperature (22 ± 4°C); only sufficient antigen for the day's tests should be removed from the refrigerator.

ii) Shake the sample well. Place 80 µl of each serum sample on a glass plate marked in 4 × 4 cm squares

iii) Shake the antigen bottle well, but gently, and place 30 µl of antigen near each serum spot.

iv) Immediately after the last drop of antigen has been added to the plate, mix the serum and antigen thoroughly (using a clean glass or plastic rod for each test) to produce a circular zone approximately 3 cm in diameter.

v)   After the initial mixing, the plate should be rotated three times in a tilting motion to ensure even dispersion of the reagents, and then incubated for 4 minutes in a humid chamber at ambient temperature

vi) The plate should be removed and rotated as above, and then returned for a second 4-minute incubation

vii) Read for agglutination immediately after the 8-minute period is completed. Any visible reaction is considered to be positive. A control serum that gives a minimum positive reaction should be tested before each day's tests are begun to verify the sensitivity of test conditions.

Like the RBT, the test is very sensitive, especially for detection of vaccine-induced antibody, and positive samples should be retested using a confirmatory and/or complementary test(s). False-negative reactions may occur, usually due to prozoning, which may be overcome by diluting the serum or retesting after a given time.

b) Complement fixation test (a prescribed test for international trade)

The CFT is widely used and accepted as a confirmatory test although it is complex to perform, requiring good laboratory facilities and adequately trained staff to accurately titrate and maintain the reagents. There are numerous variations of the CFT in use, but this test is most conveniently carried out in a microtitre format. Either warm or cold fixation may be used for the incubation of serum, antigen and complement: either 37°C for 30 minutes or 4°C for 14–18 hours. A number of factors affect the choice of the method: anti-complementary activity in serum samples of poor quality is more evident with cold fixation, while fixation at 37°C increases the frequency and intensity of prozones, and a number of dilutions must be tested for each sample.

Several methods have been proposed for the CFT using different concentrations of fresh or preserved sheep red blood cells (SRBCs) (a 2, 2.5% or 3% suspension is usually recommended) sensitised with an equal volume of rabbit anti-SRBC serum diluted to contain several times (usually from two to five times) the minimum concentration required to produce 100% lysis of SRBCs in the presence of a titrated solution of guinea-pig complement. The latter is independently titrated (in the presence or absence of antigen according to the method) to determine the amount of complement required to produce either 50% or 100% lysis of sensitised SRBCs in a unit volume of a standardised suspension; these are defined as the 50% or 100% haemolytic unit of complement/minimum haemolytic dose (C'H or MHD_{50} or C'H or MHD_{100}), respectively. It is generally recommended to titrate the complement before each set of tests, a macromethod being preferred for an optimal determination of $C'H_{50}$. Usually, 1.25–2 $C'H_{100}$ or 5–6 $C'H_{50}$ are used in the test.

Barbital (veronal) buffered saline is the standard diluent for the CFT. This is prepared from tablets available commercially; otherwise it may be prepared from a stock solution of sodium chloride (42.5 g), barbituric acid (2.875 g), sodium diethyl barbiturate (1.875 g), magnesium sulphate (1.018 g), and calcium chloride (0.147 g) in 1 litre of distilled water and diluted by the addition of four volumes of 0.04% gelatin solution before use.

- **Antigen production**

Numerous variations of the test exist but, whichever procedure is selected, the test must use an antigen that has been prepared from an approved smooth strain of *B. abortus*, such as S99 or S1119-3, and standardised against the OIEISS. Antigen for the CFT can be prepared by special procedures (1, 34) or a whole cell antigen can be used after diluting the stock suspension such that the PCV of the concentrated antigen suspension for CFT should be approximately 2% before standardisation against the OIEISS. The

antigen should be standardised to give 50% fixation at a dilution of 1/200 of the OIEISS and must also show complete fixation at the lower serum dilutions, because too weak (or too strong) a concentration of antigen may not produce 100% fixation at the lower dilutions of serum. When two dilutions of antigen are suitable, the more concentrated antigen suspension must be chosen in order to avoid prozone occurrence.

The appearance of the antigen when diluted 1/10 must be that of a uniform, dense, white suspension with no visible aggregation or deposit after incubation at 37°C for 18 hours. It must not produce anti-complementary effects at the working strength for the test. The antigen is stored at 4°C and should not be frozen.

- **Test procedure (example)**

The undiluted test sera and appropriate working standards should be inactivated for 30 minutes in a water bath at 60°C ± 2°C. If previously diluted with an equal volume of veronal buffered saline these sera could be inactivated at 58°C ± 2°C for 50 minutes. Usually, only one serum dilution is tested routinely (generally 1/4 or 1/5 depending on the CF procedure chosen), but serial dilutions are recommended for trade purposes in order to detect prozone.

Using standard 96-well microtitre plates with round (U) bottoms, the technique is usually performed as follows:

i) Volumes of 25 µl of diluted inactivated test serum are placed in the well of the first, second and third rows. The first row is an anti-complementary control for each serum. Volumes of 25 µl of CFT buffer are added to the wells of the first row (anti-complementary controls) to compensate for lack of antigen. Volumes of 25 µl of CFT buffer are added to all other wells except those of the second row. Serial doubling dilutions are then made by transferring 25 µl volumes of serum from the third row onwards; 25 µl of the resulting mixture in the last row are discarded.

ii) Volumes of 25 µl of antigen, diluted to working strength, are added to each well except in the first row.

iii) Volumes of 25 µl of complement, diluted to the number of units required, are added to each well.

iv) Control wells containing diluent only, complement + diluent, antigen + complement + diluent, are set up to contain 75 µl total volume in each case. A control serum that gives a minimum positive reaction should be tested in each set of tests to verify the sensitivity of test conditions.

v) The plates are incubated at 37°C for 30 minutes or at 4°C overnight, and a volume (25 or 50 µl according to the technique) of sensitised SRBCs is added to each well. The plates are re-incubated at 37°C for 30 minutes.

vi) The results are read after the plates have been centrifuged at 1000 ***g*** for 10 minutes at 4°C or left to stand at 4°C for 2–3 hours to allow unlysed cells to settle. The degree of haemolysis is compared with standards corresponding to 0, 25, 50, 75 and 100% lysis. The absence of anti-complementary activity is checked for each serum in the first row.

vii) Standardisation of results of the CFT:

There is a unit system that is based on the OIEISS. This serum contains 1000 ICFTU (international complement fixation test units) per ml. If this serum is tested in a given method and gives a titre of, for example 200 (50% haemolysis), then the factor for an unknown serum tested by that method can be found from the formula: $1000 \times 1/200 \times$ titre of test serum = number of ICFTU of antibody in the test serum per ml. The OIEISS contains specific IgG; national standard sera should also depend on this isotype for their specific complement-fixing activity. Difficulties in standardisation arise because different techniques selectively favour CF by different immunoglobulin isotypes. It is recommended that any country using the CFT on a national scale should obtain agreement among the different laboratories performing the test to use the same method in order to obtain the same level of sensitivity. To facilitate comparison between countries, results should always be expressed in ICFTUs, calculated in relation to those obtained in a parallel titration with a standard serum, which in turn may be calibrated against the OIEISS.

vii) *Interpretation of the results:* Sera giving a titre equivalent to 20 ICFTU/ml or more are considered to be positive.

This procedure is an example, other volumes and quantities of reagents could be chosen provided that the test is standardised against the OIEISS as described above and the results expressed in ICFTU/ml.

The CFT is usually very specific. However, like all other serological tests, it could sometimes give a positive result due to S19 vaccination or due to FPSR. Therefore positive reactions should be investigated using suitable confirmatory and/or complementary strategies. Females that have been vaccinated with *B. abortus* S19 between 3 and 6 months are usually considered to be positive if the sera give positive fixation at a titre of 30 or greater ICFTU/ml when the animals are tested at an age of 18 months or older.

c) **Enzyme-linked immunosorbent assays (prescribed tests for international trade)**

- **Indirect ELISA**

Numerous variations of the indirect ELISA (I-ELISA) have been described employing different antigen preparations, antiglobulin-enzyme conjugates, and substrate/chromogens. Several commercial I-ELISAs using whole cell, smooth lipopolysaccharide (sLPS) or the O-polysaccharide (OPS) as antigens that have been validated in extensive field trials are available and are in wide use. In the interests of international harmonisation, the three OIE ELISA Standard Sera should be used by national reference laboratories to check or calibrate the particular test method in question.

These assays should be calibrated such that the optical density (OD) of the strong positive OIE ELISA Standard Serum should represent a point on the linear portion of a typical dose–response curve just below the plateau. The weak positive OIE ELISA Standard Serum should consistently give a positive reaction that lies on the linear portion of the same dose–response curve just above the positive/negative threshold. The negative serum and the buffer control should give reactions that are always less than the positive/negative threshold (96). Finally the cut-off should be established in the test population using appropriate validation techniques (see Chapter 1.1.4 Principles of validation of diagnostic assays for infectious diseases).

The I-ELISAs that use sLPS or OPS as antigens are highly sensitive for the detection of anti-*Brucella* antibodies, but are not capable of fully resolving the problem of differentiating between antibodies resulting from S19 vaccination.

The problem with FPSR may be partly overcome by performing I-ELISAs using rough LPS (rLPS) or cytosol antigens. Most FPSR are a result of cross reaction with the OPS portion of the sLPS molecule, however, cross reaction among core regions of LPS are less frequent (63, 64).

For the screening I-ELISA, preparations rich in sLPS or OPS should be used as the optimal antigen. There are several protocols for preparing a suitable antigen.

Monoclonal, polyclonal antiglobulin or protein G or AG enzyme conjugates may be used depending on availability and performance requirements. An MAb specific for the heavy chain of bovine IgG$_1$ may provide some improvement in specificity at the possible cost of some loss of sensitivity while a protein G or AG enzyme conjugate may provide a reagent useful for testing a variety of mammalian species (55, 63).

The test method described below is an example of a test that has been internationally validated and has been used extensively in internationally sponsored, technical cooperation and research collaboration projects world-wide.

The antigen-coating buffer is 0.05 M carbonate/bicarbonate buffer, pH 9.6, composed of sodium hydrogen carbonate (2.93 g) and sodium carbonate (1.59 g) (sodium azide [0.20 g/litre] is optional) in 1 litre of distilled water. The conjugate and test sera diluent buffer is 0.01 M PBS, pH 7.2, composed of disodium hydrogen orthophosphate (1.4 g), potassium dihydrogen phosphate (0.20 g), sodium chloride (8.50 g) and 0.05% Tween 20 dissolved in 1 litre of distilled water (PBST). This buffer is also used as wash buffer.

The conjugate used in this example is an MAb specific for the heavy chain of bovine IgG$_1$ and conjugated to horseradish peroxidase (HRPO). The substrate stock solution is 3% hydrogen peroxide. The chromogen stock solution is 0.16 M 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) in distilled water. Substrate buffer is citrate buffer, pH 4.5, composed of trisodium citrate dihydrate (7.6 g) and citric acid (4.6 g) dissolved in 1 litre of distilled water. The enzymatic reaction-stopping solution is 4% sodium dodecyl sulphate (SDS).

- **Antigen production (example)**

sLPS from *B. abortus* S1119-3 or S99 is extracted by heating 5 g dry weight (or 50 g wet weight) of cells suspended in 170 ml distilled water to 66°C followed by the addition of 190 ml of 90% (v/v) phenol at 66°C. The mixture is stirred continuously at 66°C for 15 minutes, cooled and centrifuged at 10,000 *g* for 15 minutes at 4°C. The brownish phenol in the bottom layer is removed with a long cannula and large cell debris may be removed by filtration (using a Whatman No. 1 filter) if necessary.

The sLPS is precipitated by the addition of 500 ml cold methanol containing 5 ml methanol saturated with sodium acetate. After 2 hours' incubation at 4°C, the precipitate is removed by centrifugation at 10,000 *g* for 10 minutes. The precipitate is stirred with 80 ml of distilled water for 18 hours and centrifuged at 10,000 *g* for 10 minutes. The supernatant solution is kept at 4°C. The precipitate is resuspended in 80 ml distilled water and stirred for an additional 2 hours at 4°C. The supernatant solution is recovered by centrifugation as above and pooled with the previously recovered supernatant.

Next, 8 g of trichloroacetic acid is added to the 160 ml of crude LPS. After stirring for 10 minutes, the precipitate is removed by centrifugation and the translucent supernatant solution is dialised against distilled water (two changes of at least 4000 ml each) and then freeze dried.

The freeze-dried LPS is weighed and reconstituted to 1 mg/ml in 0.05 M carbonate buffer, pH 9.6, and sonicated in an ice bath using approximately 6 watts three times for 1 minute each. The LPS is then freeze dried in 1 ml amounts and stored at room temperature.

- **Test procedure (example)**

i)   The freeze-dried sLPS is reconstituted to 1 ml with distilled water and is further diluted 1/1000 (or to a dilution predetermined by titration against the OIE ELISA Standard Sera) in 0.05 M carbonate buffer, pH 9.6. To coat the microplates, 100 µl volumes of the diluted sLPS solution are added to all wells, and the plates are covered and incubated for 18 hours at 4°C. After incubation, the plates may be used or sealed, frozen and stored at –20°C for up to a year. Frozen plates are thawed for 30–45 minutes at 37°C before use.

ii) Unbound antigen is removed by washing all microplate wells with PBST four times. Volumes (100 µl) of serum diluted in the range of 1/50 to 1/200 in PBST, pH 6.3, containing 7.5 mM each of EDTA and ethylene glycol tetra-acetic acid (EGTA) (PBST/EDTA) are added to specified wells and incubated at ambient temperature for 30 minutes.

iii) Test sera are added to the plates and may be tested singly or in duplicate. The controls, calibrated against the OIE ELISA Standard Sera, are set up in duplicate wells and include a strong positive, a weak positive, a negative control serum, and a buffer control.

iv) Unbound serum is removed by washing four times with PBST (PBST containing EDTA/EGTA must not be used with HRPO as it inactivates the enzyme). Volumes (100 µl) of conjugate (MAb M23) specific for a heavy chain epitope of bovine IgG_1 conjugated with HRPO and diluted in PBST (predetermined by titration) are added to each well and the plates are incubated at ambient temperature for 30 minutes.

v)   Unbound conjugate is removed by four washing steps. Volumes (100 µl) of substrate/chromogen (1.0 mM H_2O_2 [100 µl/20 ml citrate buffer] and 4 mM ABTS [500 µl/20 ml citrate buffer]) are added to each well, the plate is shaken for 10 minutes and colour development is assessed in a spectrophotometer at 414 or 405 nm. If required, 100 µl volumes of 4% SDS may be added directly to all wells as a stopping reagent.

vi) The control wells containing the strong positive serum are considered to be 100% positive and all data are calculated from these absorbance readings (between 1.000 and 1.800) using the equation:

Per cent positivity (%P) = absorbance (test sample)/absorbance (strong positive control) × 100

The sLPS antigen, small amounts of the MAb specific for the heavy chain of bovine IgG_1, software for generation of data using particular spectrophotometers and a standard test protocol for the I-ELISA are available for research and standardisation purposes[3].

Using this or another similar I-ELISA calibrated against the OIE ELISA Standard Sera described above, the diagnostic sensitivity should be equal to or greater than that of the BBATs (RBT/BPAT) in the testing of infected cattle. However, like all other serological tests, it could give a positive result because of S19 vaccination or FPSR. Positive reactions should be investigated using suitable confirmatory and/or complementary strategies as for CFT.

- **Competitive ELISA**

The competitive ELISA (C-ELISA) using an MAb specific for one of the epitopes of the *Brucella* sp. OPS has been shown to have higher specificity but lower sensitivity than the I-ELISA (47, 55, 60, 80, 89). This is accomplished by selecting an MAb that has higher affinity than cross-reacting antibody. However, it has been shown that the C-ELISA eliminates some but not all reactions (FPSR) due to cross-reacting bacteria (55, 57).The C-ELISA is also capable of eliminating most reactions due to residual antibody produced in response to vaccination with S19. The choice of MAb and its unique specificity and affinity will have a distinct influence on the diagnostic performance characteristics of the assay. As with any MAb-based assay, the universal availability of the MAb or the hybridoma must also be considered with respect to international acceptance and widespread use.

Several variations of the C-ELISA have been described including antigens prepared from different smooth *Brucella* strains. The C-ELISA is also commercially available. Some protocols are less sensitive than others, therefore results obtained from different assays are not always comparable. In the interests of international

---

3   Obtainable from the OIE Reference Laboratory for Brucellosis at the Animal Diseases Research Institute, 3851 Fallowfield Road, Nepean, Ontario K2H 8P9, Canada.

harmonisation, the three OIE ELISA Standard Sera should be used by national reference laboratories to check or calibrate the test method in question.

The assay should be calibrated such that the OD of the strong positive OIE ELISA Standard Serum should represent a point on the linear portion of a typical dose–response curve just above the plateau (i.e. close to maximal inhibition). The weak positive OIE ELISA Standard Serum should give a reaction that lies on the linear portion of the same dose–response curve just above the positive/negative threshold (i.e. moderate inhibition). The negative serum and the buffer/MAb control should give reactions that are always less than the positive/negative threshold (i.e. minimal inhibition). Moreover, the cut-off should be established in the test population with appropriate validation techniques (see Chapter 1.1.4 Principles of validation of diagnostic assays for infectious diseases).

The test method described below is an example of a test, which has been internationally validated and has been used extensively in internationally sponsored, technical cooperation and research collaboration projects world-wide.

The buffer systems are the same as those described for the I-ELISA.

- **Antigen production (example)**

sLPS from *B. abortus* S1119-3 is prepared and used as for the I-ELISA.

- **Test procedure**

i)   The freeze-dried sLPS is reconstituted to 1 ml with distilled water and further diluted 1/1000 with 0.05 M carbonate buffer, pH 9.6. To coat the microplates, 100 µl volumes of LPS solution are added to all wells and the plates are covered and incubated for 18 hours at 4°C. After incubation, the plates may be used or sealed, frozen and stored at –20°C for up to 1 year. Frozen plates are thawed for 30–45 minutes at 37°C before use.

ii) Unbound antigen is removed by washing all microplate wells four times with PBST. Volumes (50 µl) of MAb (M84 in this example) diluted appropriately in PBST/EDTA are added to each well, followed immediately by 50 µl volumes of serum diluted 1/10 in PBST/EDTA. Plates are incubated for 30 minutes at ambient temperature with shaking for at least the initial 3 minutes.

Iii) Test sera are added to the plates and may be tested as singly or in duplicate. The controls, calibrated against the OIE ELISA Standard Sera, are set up in duplicate wells and include a strong positive, a weak positive, a negative control serum, and a buffer control.

iv) Unbound serum and MAb are removed by washing the microplate four times with PBST. Volumes (100 µl) of commercial goat anti-mouse IgG (H and L chain) HRPO conjugate diluted in PBST (predetermined by titration) are added to each well and the plates are incubated at ambient temperature for 30 minutes.

v)   Unbound conjugate is removed by four washing steps. Volumes (100 µl) of substrate/chromogen (1.0 mM H_2O_2 and 4 mM ABTS) are added to each well, the plates are shaken for 10 minutes and colour development is assessed in a spectrophotometer at 414 or 405 nm. If required, 100 µl volumes of 4% SDS may be added directly as a stopping reagent.

vi) The control wells containing MAb and buffer (no serum) are considered to give 0% inhibition and all data are calculated from these absorbance readings (between 1.000 and 1.800) using the equation:

Per cent inhibition (%I) = 100 – (absorbance [test sample]/absorbance [buffer control] × 100)

The sLPS antigen, small amounts of the MAb, software for generation of data using particular spectrophotometers and a standard operating procedure for the C-ELISA are available for research and standardisation (see footnote 3 for address).

Using this or a similar C-ELISA protocol calibrated against the OIE ELISA Standard Sera, the diagnostic sensitivity could be equivalent to the BBATs and the I-ELISAs in the testing of infected cattle (59, 60, 62). However, like all other serological tests, it could give a positive result because of S19 vaccination or FPSR. Positive reactions should be investigated using suitable confirmatory and/or complementary strategies as for CFT.

d)   Fluorescence polarisation assay (a prescribed test for international trade)

The FPA is a simple technique for measuring antigen/antibody interaction and may be performed in a laboratory setting or in the field. It is a homogeneous assay in which analytes are not separated and it is therefore very rapid.

The mechanism of the assay is based on random rotation of molecules in solution. Molecular size is the main factor influencing the rate of rotation, which is inversely related. Thus a small molecule rotates faster

than a large molecule. If a molecule is labelled with a fluorochrome, the time of rotation through an angle of 68.5° can be determined by measuring polarised light intensity in vertical and horizontal planes. A large molecule emits more light in a single plane (more polarised) than a small molecule rotating faster and emitting more depolarised light.

For most FPAs, an antigen of small molecular weight, less than 50 kD, is labelled with a fluorochrome and added to serum or other fluid to be tested for the presence of antibody. If antibody is present, attachment to the labelled antigen will cause its rotational rate to decrease and this decrease can be measured.

For the diagnosis of brucellosis, a small molecular weight fragment (average 22 kD) of the OPS of *B. abortus* strain 1119-3 sLPS is labelled with fluorescein isothiocyanate (FITC) and used as the antigen. This antigen is added to diluted serum or whole blood and a measure of the antibody content is obtained in about 2 minutes (for serum) or 15 seconds (for blood) after the addition of antigen using a fluorescence polarisation analyser (58, 62).

The FPA can be performed in glass tubes or a 96-well plate format. The bovine serum is diluted 1/10 for the plate test or 1/100 for the tube test; if EDTA-treated blood is used the dilution for the tube test is 1/50 and 1/5 for the plate test (heparin-treated blood tends to increase assay variability). The diluent used is 0.01 M Tris (1.21 g), containing 0.15 M sodium chloride (8.5 g), 0.05% Igepal CA630 (500 µl) (formerly NP40), 10 mM EDTA (3.73 g) per litre of distilled water, pH 7.2 (Tris buffer). An initial reading to assess light scatter is obtained with the fluorescence polarisation analyser (FPM) after mixing. Suitably labelled titrated antigen (usually giving an intensity of 250,000–300,000) is added, mixed and a second reading is obtained in the FPM about 2 minutes later for serum and 15 seconds for blood. A reading (in millipolarisation units, mP) over the established threshold level is indicative of a positive reaction. A typical threshold level is 90–100 mP units, however, the test should be calibrated locally against International Standard reference sera (the expected values are pending). Control sera of strong positive, weak positive and negative, as well as S19 vaccinate serum, should be included.

- **Antigen production (example)**

OPS from 5 g dry weight (or 50 g wet weight) of *B. abortus* S1119-3 is prepared by adding 400 ml of 2% (v/v) acetic acid, autoclaving the suspension for 15 minutes at 121°C and removing the cellular debris by centrifugation at 10,000 *g* for 10 minutes at 4°C. The supernatant solution is then treated with 20 g of trichloroacetic acid to precipitate any proteins and nucleic acids. The precipitate is again removed by centrifugation at 10,000 *g* for 10 minutes at 4°C. The supernatant fluid is dialysed against at least 100 volumes of distilled water and freeze dried.

3 mg of OPS are dissolved in 0.6 ml of 0.1 M sodium hydroxide (4 g NaOH/litre) and incubated at 37°C for 1 hour, followed by the addition of 0.3 ml of FITC isomer 1 at a concentration of 100 mg/ml in dimethyl sulphoxide and a further incubation at 37°C for 1 hour. The conjugated OPS is applied to a 1 × 10 cm column packed with DEAE (diethylaminoethyl) Sephadex A 25 equilibrated in 0.01 M phosphate buffer, pH 7.4. The first fraction (after 10–15 ml of buffer) is bright green, after which the buffer is switched to 0.1 M phosphate, pH 7.4. This results in the elution of 10–15 ml of buffer followed by 25–40 ml of green fluorescent material. The latter material is the antigen used in the FPA. Antigen preparation may be scaled up proportionally.

The amount of antigen used per test is determined by diluting the material derived above until a total fluorescence intensity of 250,000–300,000 is achieved using the FPM.

The antigen can be stored as a liquid for several years at 4°C in a dark bottle or it may be freeze dried in dark bottles.

Small quantities of labelled antigen for research and standardisation purposes and standard operating procedures for antigen preparation and the FPA may be obtained (see footnote 3 for address).

- **Test procedure**

i) 1 ml of Tris buffer is added to a 10 × 75 mm borosilicate glass tube followed by 10 µl of serum or 20 µl of EDTA-treated blood. For the 96-well format, 20 µl of serum is added to 180 µl of buffer. It is important to mix well. A reading is obtained on the FPM to determine light scatter.

ii) A volume of antigen, which results in a total fluorescence intensity of $250–300 \times 10^3$, is added to the tube and mixed well. This volume will vary from batch to batch, but is generally in the range of about 10 µl. A second reading is obtained on the FPM after incubation at ambient temperature for approximately 2 minutes for serum and 15 seconds for EDTA-treated blood.

iii) A reading above the predetermined threshold is indicative of a positive reaction.

iv) The following are included in each batch of tests: a strong positive, a weak positive, a negative working standard serum (calibrated against the OIE ELISA Standard Sera).

The diagnostic sensitivity and specificity of the FPA for bovine brucellosis are almost identical to those of the C-ELISA. The diagnostic specificity for cattle recently vaccinated with S19 is over 99% (58). However the specificity of FPA in FPSR conditions is currently unknown. Like all other serological tests, positive reactions should be investigated using suitable confirmatory and/or complementary strategies. The FPA should be standardised such that the OIE ELISA strong positive and weak positive sera consistently give positive results. Moreover, the cut-off should be established in the test population with appropriate validation techniques (see Chapter 1.1.4 Principles of validation of diagnostic assays for infectious diseases).

3. Other tests

a) Brucellin skin test

An alternative immunological test is the brucellin skin test, which can be used for screening unvaccinated herds, provided that a purified (free of sLPS) and standardised antigen preparation (e.g. brucellin INRA) is used.

The brucellin skin test has a very high specificity, such that serologically negative unvaccinated animals that are positive reactors to the brucellin test should be regarded as infected animals (70, 73). Also, results of this test may aid the interpretation of serological reactions thought to be FPSR due to infection with cross-reacting bacteria, especially in brucellosis-free areas (20, 70, 73).

Not all infected animals react, therefore this test alone cannot be recommended as the sole diagnostic test or for the purposes of international trade.

It is essential to use a standardised, defined brucellin preparation that does not contain sLPS antigen, as this may provoke nonspecific inflammatory reactions or interfere with subsequent serological tests. One such preparation is brucellin INRA prepared from a rough strain of *B. melitensis* that is commercially available[4].

- **Test procedure**

  i) A volume of 0.1 ml of brucellin is injected intradermally into the caudal fold, the skin of the flank, or the side of the neck.

  ii) The test is read after 48–72 hours.

  iii) The skin thickness at the injection site is measured with vernier callipers before injection and at re-examination.

  iv) A strong positive reaction is easily recognised by local swelling and induration. However, borderline reactions require careful interpretation. Skin thickening of 1.5–2 mm would be considered as a positive reaction.

Although the brucellin intradermal test is one of the most specific tests in brucellosis (in unvaccinated animals), diagnosis should not be made solely on the basis of positive intradermal reactions given by a few animals in the herd, but should be supported by a reliable serological test. The intradermal inoculation of brucellin might induce a temporary anergy in the cellular immune response. Therefore an interval of 6 weeks is generally recommended between two tests on the same animal.

b) Serum agglutination test

While not recognised as a prescribed or alternative test, the SAT has been used with success for many years in surveillance and control programmes for bovine brucellosis. Its specificity is significantly improved with the addition of EDTA to the antigen (30, 45, 61).

The antigen represents a bacterial suspension in phenol saline (NaCl 0.85% [w/v] and phenol at 0.5% [v/v]). Formaldehyde must not be used. Antigens may be delivered in the concentrated state provided the dilution factor to be used is indicated on the bottle label. EDTA may be added to the antigen suspension to 5 mM final test dilution to reduce the level of false-positive results. Subsequently the pH of 7.2 must be readjusted in the antigen suspension.

The OIEISS contains 1000 IUs of agglutination. The antigen should be prepared without reference to the cell concentration, but its sensitivity must be standardised in relation to the OIEISS in such a way that the antigen produces either 50% agglutination with a final serum dilution of 1/600 to 1/1000 or 75% agglutination with a final serum dilution of 1/500 to 1/750. It may also be advisable to compare the reactivity of new and previously standardised batches of antigen using a panel of defined sera.

---

4   Brucellergène OCB®, Synbiotics Europe, 2 rue Alexander Fleming, 69007 Lyon, France.

The test is performed either in tubes or in microplates. The mixture of antigen and serum dilutions should be incubated for 16–24 hours at 37°C. If the test is carried out in microplates, the incubation time can be shortened to 6 hours. At least three dilutions must be prepared for each serum in order to refute prozone negative responders. Dilutions of suspect serum must be made in such a way that the reading of the reaction at the positivity limit is made in the median tube (or well for the microplate method).

Interpretation of results: The degree of *Brucella* agglutination in a serum must be expressed in IU per ml. A serum containing 30 or more IU per ml is considered to be positive.

c) Native hapten and cytosol protein-based tests

Native hapten tests[5] are highly specific in S19 vaccination contexts, and have been used successfully in an eradication programme in combination with the RBT as a screening test (3). The optimal sensitivity (close to that of CFT but lower than that of RBT and sLPS-based I-ELISAs) is obtained in a reverse radial immunodiffusion (RID) system in which the serum diffuses into a hypertonic gel containing the polysaccharide (21, 40). However, the double gel diffusion procedure is also useful (43, 44). Calves vaccinated subcutaneously with the standard dose of S19 at 3–5 months of age are negative 2 months after vaccination, and adult cattle vaccinated subcutaneously 4–5 months previously with the reduced dose of S19 do not give positive reactions unless the animals become infected and shed the vaccine in their milk (40). The conjunctival vaccination (both in young and adults) reduces the time to obtain a negative response in native hapten tests. A remarkable characteristic of the RID test is that a positive result correlates with *Brucella* shedding as shown in experimentally infected cattle and in naturally infected cattle undergoing antibiotic treatment (39). Precipitin tests using native hapten or *Brucella* cytosol proteins have also been shown to eliminate, in most cases, FPSR reactions caused by *Yersinia enterocolitica* O:9 and FPSR of unknown origin (55).

d) Milk tests

An efficient means of screening dairy herds is by testing milk from the bulk tank. It should be borne in mind that in the last period of gestation, pregnant cows are dried and do not participate in the bulk tank sample. In contrast, these animals, if infected, are most likely to be positive by serological diagnosis. Therefore, immediately after parturition, bulk tank should be re-tested. Milk from these sources can be obtained cheaply and more frequently than blood samples and is often available centrally at dairies. When a positive test result is obtained, all cows contributing milk should be blood tested. The milk I-ELISA is a sensitive and specific test, and is particularly valuable for testing large herds. The milk ring test (MRT) is a suitable alternative if the ELISA is not available.

- **Milk I-ELISA**

As with the serum I-ELISA numerous variations of the milk I-ELISA are in use. Several commercial I-ELISAs are available that have been validated in extensive field trials and are in wide use. In the interests of international harmonisation, the three OIE ELISA Standard Sera should be used by national reference laboratories to check or calibrate the particular test method in question. The I-ELISA should be standardised such that the OIE ELISA strong positive standard when diluted 1/125 in negative serum and further diluted 1/10 in negative milk consistently tests positive. Bulk milk samples are generally tested at much lower dilutions than sera, i.e. undiluted to 1/2 to 1/10 in diluent buffer, with the remainder of the assay being similar to that described for serum. The C-ELISA should not be used to test whole milk but may be used with whey samples.

- **Milk ring test**

In lactating animals, the MRT can be used for screening herds for brucellosis. In large herds (> 100 lactating cows), the sensitivity of the test becomes less reliable. The MRT may be adjusted to compensate for the dilution factor from bulk milk samples from large herds. The samples are adjusted according to the following formula: herd size < 150 animals use 1 ml bulk milk, 150–450 use 2 ml milk sample, 451–700 use 3 ml milk sample. False-positive reactions may occur in cattle vaccinated less than 4 months prior to testing, in samples containing abnormal milk (such as colostrum) or in cases of mastitis. Therefore, it is not recommended to use this test in very small farms where these problems have a greater impact on the test results.

- **Antigen production**

MRT antigen is prepared from concentrated, killed *B. abortus* S99 or S1119-3 cell suspension, grown as described previously. It is centrifuged at, for example, 23,000 *g* for 10 minutes at 4°C, followed by resuspension in haematoxylin-staining solution. Various satisfactory methods are in use; one example is as follows: 100 ml of 4% (w/v) haematoxylin (CI No. 75290) dissolved in 95% ethanol is added to a solution of

---

5 The detailed procedure can be obtained from the Brucellosis Laboratory, Centro de Investigacion y Tecnologia Agroalimentaria/Gobierno de Aragon, Avenida Montaňana 930, 50059 Zaragoza, Spain.

ammonium aluminium sulphate (5 g) in 100 ml of distilled water and 48 ml of glycerol. 2 ml of freshly prepared 10% (w/v) sodium iodate is added to the solution. After standing for 30 minutes at room temperature, the deep purple solution is added to 940 ml of 10% (w/v) ammonium aluminium sulphate in distilled water. The pH of this mixture is adjusted to 3.1, and the solution must be aged by storage at room temperature in the dark for 45–90 days.

Before use, the staining solution is shaken and filtered through cotton wool. The packed cells are suspended in the staining solution at the rate of 1 g per 30 ml stain, and held at room temperature for 48 hours (some laboratories prefer to heat at 80°C for 10 minutes instead). The stained cells are then deposited by centrifugation, and washed three times in a solution of sodium chloride (6.4 g), 85% lactic acid (1.5 ml) and 10% sodium hydroxide (4.4 ml) in 1.6 litres of distilled water, final pH 3.0. The washed cells are resuspended at the rate of 1 g in 27 ml of a diluent consisting of 0.5% phenol saline, adjusted to pH 4.0 by the addition of 0.1 M citric acid (approximately 2.5 ml) and 0.5 M disodium hydrogen phosphate (approximately 1 ml) and maintained at 4°C for 24 hours. The mixture is filtered through cotton wool, the pH is checked, and the PCV is determined and adjusted to approximately 4%.

The sensitivity of the new batch should be compared with a previously standardised batch using a panel of samples of varying degrees of reaction prepared by diluting a positive serum in milk. The antigen should be standardised against the OIEISS so that a 1/500 dilution is positive and 1/1000 dilution is negative. The antigen should be stored as recommended by the manufacturer but usually should be stored at 4°C.

The pH of the antigen should be between 3.3 and 3.7 and its colour should be dark blue. A little free stain in the supernatant of a centrifuged sample is permissible. When diluted in milk from a brucellosis-free animal, the antigen must produce a uniform coloration of the milk layer with no deposit and no coloration of the cream layer.

- **Test procedure**

The test is performed on bulk tank milk samples. If necessary, samples could be pretreated with preservative (0.1% formalin or 0.02% bronopol) for 2–3 days at 4°C prior to use.

i) Bring the milk samples and antigen to room temperature (20 ± 3°C); only sufficient antigen for the day's tests should be removed from the refrigerator.

ii) Gently shake the antigen bottle well.

iii) The test is performed by adding 30–50 µl of antigen to a 1–2 ml volume of whole milk (the volume of milk may be increased for bulk samples from larger herds – see above "Milk ring test").

iv) The height of the milk column in the tube must be at least 25 mm. The milk samples must not have been frozen, heated, subjected to violent shaking or stored for more that 72 hours.

v) The milk/antigen mixtures are normally incubated at 37°C for 1 hour, together with positive and negative working standards. However, overnight incubation at 4°C increases the sensitivity of the test and allows for easier reading.

vi) A strongly positive reaction is indicated by formation of a dark blue ring above a white milk column. Any blue layer at the interface of milk and cream should be considered to be positive as it might be significant, especially in large herds.

vii) The test is considered to be negative if the colour of the underlying milk exceeds that of the cream layer.

viii) When the MRT is adjusted for large herd sizes (2 or 3 ml of milk used), 0.1 ml of pooled negative cream is added to the test tube and is followed by 30–50 µl of the ring test antigen. After mixing, the test is incubated and read in the same manner as the unadjusted MRT. The negative pooled cream is collected from the separation of composite, unpasteurised milk from a brucellosis negative herd of 25 or more cows.

e) Interferon gamma test

As the prevalence of brucellosis decreases, accuracy of serological tests becomes more important. False-positive reactions result in trace-backs and epidemiological investigations that are expensive and time consuming. Therefore, assays that eliminate FPSR will become more and more useful. In general, the interferon gamma test involves stimulation of lymphocytes in whole blood with a suitable antigen, in this case, Brucellin has been shown to work well and then measuring the resulting gamma interferon production by a capture ELISA (41, 90, 91). This test could be useful in the discrimination of FPSR but more specific antigens are needed and the protocol needs to be standardised.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

As mentioned previously, brucellosis is one of the most easily acquired laboratory infections, and strict safety precautions should be observed. Laboratory manipulation of live cultures of *Brucella,* including vaccine strains, is hazardous and must be done under containment level 3 or higher, as outlined in Chapter 1.1.2, to minimise occupational exposure.

C1. Brucellin

Brucellin–INRA is an LPS-free extract from rough *B. melitensis* B115. This preparation does not provoke formation of antibodies reactive in BBAT, CFT or ELISA.

1. Seed management

a) Characteristics of the seed

Production of brucellin-INRA is based on a seed-lot system as described for antigens and vaccines. The original seed *B. melitensis* strain B115 for brucellin production[6] should be propagated to produce a seed lot, which should be preserved by lyophilisation or freezing at liquid nitrogen temperature. It should conform to the properties of a pure culture of a rough strain of *B. melitensis* and must not produce smooth *Brucella* LPS. It should produce reasonable yields of a mixture of protein antigens reactive with antisera to smooth and rough *Brucella* strains.

b) Method of culture (1)

Brucella melitensis strain B115 is best grown in the liquid medium described above for fermenter culture. It may be grown by the batch or continuous method in a fermenter or in flasks agitated on a shaker. Purity checks should be made on each single harvest, and the organisms must be in the rough phase.

c) Validation as an *in-vivo* diagnostic reagent

Laboratory and field studies in France have confirmed that brucellin-INRA is safe, non-toxic and specific in action. The preparation contains 50–75% proteins, mainly of low molecular weight and 15–30% carbohydrate. It does not contain LPS antigens. Brucellin-INRA does not provoke inflammatory responses in unsensitised animals, and it is not in itself a sensitising agent. It does not provoke antibodies reactive in the standard serological tests for brucellosis. More than 90% of small ruminants infected with *B. melitensis* manifest delayed hypersensitivity to brucellin-NRA at some stage. The preparation is not recommended as a diagnostic agent for individual animals, but can be useful when used for screening herds. It is given to small ruminants in 100-µg doses by the intradermal route, and provokes a local delayed hypersensitivity reaction visible at 48–72 hours in sensitised animals. Positive reactions can be given by vaccinated as well as by infected animals (70, 73).

2. Method of manufacture (1)

Brucella melitensis B115 cells are killed after culture by raising the temperature to 70°C for 90 minutes, cooled to 4°C, and harvested by centrifugation at 9000 *g* for 15 minutes at 4°C. The cells are washed in cold sterile distilled water and dehydrated by precipitating with three volumes of acetone at –20°C, and then allowed to stand at –20°C for 24–48 hours. After repeated washing in cold acetone, followed by a final rinse in diethyl ether, the cells are dried over calcium chloride and held at 4°C. The dried cells are subjected to a viability check. They are resuspended in sterile 2.5% sodium chloride to a final concentration of 5% (w/v) and agitated for 3 days at 4°C. Bacterial cells are removed by centrifugation as above, and the supernatant is concentrated to one-fourth the volume by ultrafiltration on a Diaflo PM10 membrane (Amicon) and precipitated by the addition of three volumes of ice-cold ethanol. The mixture is held at 4°C for 24 hours and the precipitate is recovered by centrifugation, redissolved in sterile water, and dialysed to remove ethanol. After centrifugation at 105,000 *g* for 6 hours at 4°C, the supernatant material, comprising the unstandardised brucellin, is subjected to assays for protein and carbohydrate. It may be freeze-dried either as bulk material or after it has been dispensed into its final containers.

3. In-process control

The crude brucellin extract should be checked for sterility after acetone extraction, to ensure killing of *Brucella* cells, and again at the end of the process to check possible contamination. The pH and protein concentration

---

6   Obtainable from Institut National de la Recherche Agronomique (INRA), Laboratoire de Pathologie Infectieuse et Immunologie, 37380 Nouzilly, France.

should be determined, and identity tests should be performed on the bulk material before filling the final containers.

4. Batch control

a) Sterility

Allergen preparations should be checked for sterility as described in Chapter 1.1.9 Tests for sterility and freedom from contamination of biological materials.

b) Safety

Samples of brucellin from the final containers should be subjected to the standard sterility test. Brucellin preparations should also be checked for abnormal toxicity. Doses equivalent to 20 cattle doses (2 ml) should be injected intraperitoneally into a pair of normal guinea-pigs that have not been exposed previously to *Brucella* organisms or their antigens. Five normal mice are also inoculated subcutaneously with 0.5 ml of the brucellin to be examined. Animals are observed for 7 days, and there should be no local or generalised reaction to the injection.

Dermo-necrotic capacity is examined by intradermal inoculation of 0.1 ml of the product to be examined into the previously shaved and disinfected flank of three normal albino guinea-pigs that have not been exposed previously to Brucella organisms or their antigens. No cutaneous reaction should be observed. Absence of allergic and serological sensitisation is checked by intradermal inoculation of three normal albino guinea-pigs, three times every 5 days, with 0.1 ml of a 1/10 dilution of the preparation to be examined. A fourth similar injection is given, 15 days later, to the same three animals and to a control lot of three guinea-pigs of the same weight that have not been injected previously. The animals should not become seropositive to the standard tests for brucellosis (RBT, CFT) when sampled 24 hours after the last injection, and should not develop delayed hypersensitivity responses.

c) Potency

The potency of brucellin preparations is determined by intradermal injection of graded doses of brucellin into guinea-pigs that have been sensitised by subcutaneous inoculation of 0.5 ml of reference brucellin[7] in Freund's complete adjuvant from 1 to 6 months previously (the use of a live *Brucella* strain, for example Rev1 strain, is possible provided that it produces the same level of sensitisation). The erythematous reactions are read and measured at 24 hours and the titre is calculated by comparison with a reference brucellin[8]. This method is only valid for comparing brucellin preparations made according to the same protocol as the sensitising allergen. Initial standardisation of a batch of allergen and the sensitisation and titration in ruminants is described (1).

d) Duration of sensitivity

Duration of sensitivity is uncertain. Individual animals vary considerably in the degree of hypersensitivity manifested to brucellin. Animals in the very early stages of infection, or with long-standing infection, may not manifest hypersensitivity to intradermal injection.

e) Stability

The freeze-dried preparation retains full potency for several years. The liquid commercial preparation should retain potency for the recommended shelf-life.

f) Preservatives

The use of preservatives is not recommended when the preparation is freeze-dried. In the liquid form, sodium merthiolate (at most 0.1 mg/ml) may be used as a preservative. If freeze-dried, the preparation should not be reconstituted until immediately before use.

g) Precautions (hazards)

Brucellin is not toxic. Nevertheless it may provoke severe hypersensitivity reactions in sensitised individuals who are accidentally exposed to it. Care should be taken to avoid accidental injection or mucosal

---

7   A national French reference brucellin has been produced by INRA-PII (F-37380 Nouzilly, France) and is obtainable from the OIE Reference Laboratory for Brucellosis, AFSSA, 23 avenue du Général-de-Gaulle, 94706 Maisons-Alfort Cedex, France.

8   The statistical procedure can be obtained from the OIE Reference Laboratory for Brucellosis, AFSSA, 23 avenue du Général-de-Gaulle, 94706 Maisons-Alfort Cedex, France.

contamination. Used containers and injection equipment should be carefully decontaminated or disposed of by incineration in a suitable disposable container.

5. Tests on final product

a) Safety

A sterility test should be performed by the recommended method. The *in-vivo* safety tests are as those described for batch control (see Section C1.4.b). These tests on the batch may be omitted if the full test is performed on the final filling lots.

b) Potency

This is performed by injection of a single dose into guinea-pigs using the procedure described in Section C1.4.c.

C2. Vaccines

Brucella abortus strain 19 vaccine

The most widely used vaccine for the prevention of brucellosis in cattle is the *Brucella abortus* S19 vaccine, which remains the reference vaccine to which any other vaccines are compared. It is used as a live vaccine and is normally given to female calves aged between 3 and 6 months as a single subcutaneous dose of 5–8 × 10^{10} viable organisms. A reduced dose of from 3×10^8 to 3×10^9 organisms can be administered subcutaneously to adult cattle, but some animals will develop persistent antibody titres and may abort and excrete the vaccine strain in the milk (81). Alternatively, it can be administered to cattle of any age as either one or two doses of 5 × 10^9 viable organisms, given by the conjunctival route; this produces protection without a persistent antibody response and reduces the risks of abortion and excretion in milk when vaccinating adult cattle.

Brucella abortus S19 vaccine induces good immunity to moderate challenge by virulent organisms. The vaccine must be prepared from USDA-derived seed (see footnote 2 for address) and each batch must be checked for purity (absence of extraneous microorganisms), viability (live bacteria per dose) and smoothness (determination of dissociation phase). Seed lots for S19 vaccine production should be regularly tested for residual virulence and immunogenicity in mice.

Control procedures for this vaccine follow.

Brucella abortus strain RB51 vaccine

Since 1996, *B. abortus* strain RB51 has become the official vaccine for prevention of brucellosis in cattle in several countries (78). However there is disagreement in regards to how the efficiency of strain RB51 compares to protection induced by S19 in cattle (53, 52, 81, 82, 84). Each country uses slightly different methods to administer the vaccine. In the USA, calves are vaccinated subcutaneously between the ages of 4 and 12 months with 1–3.4 × 10^{10} viable strain RB51 organisms. Vaccination of cattle over 12 months of age is carried out only under authorisation from the State or Federal Animal Health Officials, and the recommended dose is 1-3 × 10^9 viable strain RB51 organisms (66, 83). In other countries, it is recommended to vaccinate cattle as calves (4–12 months of age) with a 1–3.4 × 10^{10} dose, with revaccination from 12 months of age onwards with a similar dose to elicit a booster effect and increase immunity (74, 78).

It has been reported that full doses of RB51 when administered intravenously in cattle induce severe placentitis and placental infection in most vaccinated cattle (68), and that there is excretion in milk in a relevant number of vaccinated animals. Field experience also indicates that it can induce abortion in some cases if applied to pregnant cattle. Due to these observations, vaccination of pregnant cattle should be avoided. One way to reduce the side effects of RB51 is to reduce the dose. When using the reduced dose of this vaccine (1 × 10^9 colony-forming units [CFU]), on late pregnant cattle, no abortions or placentitis lesions are produced in subcutaneously vaccinated cattle (69), but the vaccine strain can be shed by a significant proportion of vaccinated animals (81). However, this reduced dose does not protect against *B. abortus* when used as a calfhood vaccination (66), but does protect when used as an adult vaccine (67).

It should be emphasised that RB51, as well as S19, can infect humans and cause undulant fever if not treated (88, 94). There have been limited studies with RB51 in humans but it appears that the risk of developing undulant fever after exposure is low (4, 83, 88). The diagnosis of the infection produced by RB51 requires special tests not available in most hospitals. Physicians making decisions on prophylactic treatment for accidental exposure to

RB51 should be informed that this vaccine strain is highly resistant to rifampicin, one of the antibiotics of choice for treating human brucellosis.

Control procedures for this vaccine follow.

Brucella melitensis strain Rev.1 vaccine

It is not infrequent to isolate *B. melitensis* in cattle in countries with a high prevalence of this infection in small ruminants (87). There has been some debate on the protective efficacy of S19 against *B. melitensis* infection in cattle and it has been hypothesised that Rev.1 should be a more effective vaccine in these conditions. However there is very little information related to this issue (39, 85). Evidence proving that S19 is able to control *B. melitensis* at the field level is also scanty (38). No experiments have been reported showing the efficacy of Rev.1 against *B. melitensis* infection in cattle. Moreover, the safety of this vaccine is practically unknown in cattle. Until the safety of Rev.1 in cattle of different physiological status and efficacy studies against *B. melitensis* under strictly controlled conditions are performed, this vaccine should not be recommended for cattle.

1. Seed management

a) Characteristics of the seed

Brucella abortus S19 original seed for vaccine production must be obtained from the USDA (see footnote 2 for address), and used to produce a seed lot that is preserved by lyophilisation or by freezing at liquid nitrogen temperature. The properties of this seed lot must conform to those of a pure culture of a CO_2-independent *B. abortus* biovar 1 that is also sensitive to benzylpenicillin, thionin blue and i-erythritol at recommended concentrations, and that displays minimal pathogenicity for guinea-pigs.

Brucella abortus RB51 original seed for vaccine production is available commercially[9]. These companies have legal rights to the vaccine.

b) Method of culture

Brucella abortus S19 for vaccine production is grown on medium free from serum or other animal products, under conditions similar to those described above for *B. abortus* S99 or S1119-3 (1).

Brucella abortus strain RB51 follows similar culture methods.

c) Validation as a vaccine

Numerous independent studies have confirmed the value of S19 as a vaccine for protecting cattle from brucellosis. The organism behaves as an attenuated strain when given to sexually immature cattle. In rare cases, it may produce localised infection in the genital tract. Antibody responses persisting for 6 months or longer are likely to occur in a substantial proportion of cattle that have been vaccinated subcutaneously with the standard dose as adults. Some of the cattle vaccinated as calves may later develop arthropathy, particularly of the femoro-tibial joints (8, 19). The vaccine is safe for most animals if administered to calves between 3 and 6 months of age. It may also be used in adult animals at a reduced dose. It produces lasting immunity to moderate challenge with virulent *B. abortus* strains, but the precise duration of this is unknown. The length of protection against *B. melitensis* is unknown. The vaccine strain is stable and reversion to virulence is extremely rare. It has been associated with the emergence of i-erythritol-using strains when inadvertently administered to pregnant animals. The organism behaves as an attenuated strain in mice, and even large inocula are rapidly cleared from the tissues.

Reports from both experimental challenge studies and field studies remain controversial as far as the value of *B. abortus* strain RB51 in protecting cattle from brucellosis is concerned (see above). The organism is attenuated in calves but not always in adults. *Brucella abortus* strain RB51 contains minimally expressed OPS and there is no serological conversion in RBT and CFT in vaccinated animals. In addition, it has also been reported that RB51 does not induce detectable antibodies, using current testing procedures, to the OPS antigen (83). However, the presence of common core epitopes in both sLPS and OPS antigenic preparations does not allow the response to RB51 to be distinguished from the response to S strains, no matter which I-ELISA is used (48). RB51 produces immunity to moderate challenge with virulent strains, but the precise duration of this is unknown. The vaccine is very stable and no reversion to smoothness has been described *in vivo* or *in vitro*. The organism behaves as an attenuated strain in a variety of animals including mice where it is rapidly cleared from the tissues.

---

9   Colorado Serum Company, 4950 York Street, P.O. Box 16428, Denver, Colorado 80216-0428, USA; or Veterinary Technologies Corporation, 1872 Pratt Drive, Suite 1100B, Blacksburg, Virginia 24060, USA.

S19 and RB51 vaccines have some virulence for humans, and infections may follow accidental inoculation with the vaccine. Care should be taken in its preparation and handling, and a hazard warning should be included on the label of the final containers. In any case, accidental inoculations should be treated with appropriate antibiotics (see Section C2.4.g).

2. Method of manufacture

For production of S19 vaccine, the procedures described above can be used, except that the cells are collected in PBS, pH 6.3, and deposited by centrifugation or by the addition of sodium carboxymethyl cellulose at a final concentration of 1.5 g/litre. The yield from one fermenter run or the pooled cells from a batch of Roux flask cultures that have been inoculated at the same time from the same seed lot constitutes a single harvest. More than one single harvest may be pooled to form a final bulk, which is used to fill the final containers of a batch of vaccine. Before pooling, each single harvest must be checked for purity, cell concentration, dissociation and identity. A similar range of tests must be done on the final bulk, which should have a viable count of between 8 and 24×10^9 CFU/ml. Adjustments in concentration are made by the addition of PBS for vaccine to be dispensed in liquid form, or by the addition of stabiliser for lyophilised vaccine. If stabiliser is to be used, loss of viability on lyophilisation should be taken into account, and should not be in excess of 50%. The final dried product should not be exposed to a temperature exceeding 35°C during drying, and the residual moisture content should be 1–2%. The contents must be sealed under vacuum or dry nitrogen immediately after drying, and stored at 4°C.

The production process for *B. abortus* strain RB51 is very similar to the one used for S19.

3. In-process control

Brucella abortus S19 vaccine should be checked for purity and smoothness during preparation of the single harvests. The cell concentration of the bulks should also be checked. This can be done by opacity measurement, but a viable count must be performed on the final filling lots. The identity of these should also be checked by agglutination tests with antiserum to *Brucella* A antigen. The viable count of the final containers should not be less than 50×10^9 per standard subcutaneous dose (5×10^9 for conjunctival dose) after lyophilisation, if this is to be done, and at least 95% of the cells must be in the smooth phase.

Brucella abortus strain RB51 vaccine should be checked for purity and roughness during preparation of the single harvests. The cell concentration of the bulks should also be checked. A viable count must be performed on the final filling lots. The viable count of the final containers should be $1–3.4 \times 10^{10}$ viable CFU of RB51 per dose (dose of 2 ml to be applied subcutaneously) and 100% of the cells must be in the rough phase. All colonies should be negative on dot-blot assays with MAbs specific for the OPS antigen.

4. Batch control

a) Sterility

Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.9.

b) Safety

The S19 vaccine is a virulent product *per se*, and it should keep a minimal virulence to be efficient (see Section C2.4.c). However a safety test is not routinely done. If desired, when a new manufacturing process is started and when a modification in the innocuousness of the vaccine preparation is expected, it may be performed on cattle. This control should be done as follows: the test uses 12 female calves, aged 4–6 months. Six young females are injected with one or three recommended doses. Each lot of six young females are kept separately. All animals are observed for 21 days. No significant local or systemic reaction should occur. If, for a given dose and route of administration, this test gives good results on a representative batch of the vaccine, it does not have to be repeated routinely on seed lots or vaccine lots prepared with the same original seed and with the same manufacturing process. A safety test on S19 vaccine may also be performed in guinea-pigs. Groups of at least ten animals are given intramuscular injections of doses of vaccine diluted in PBS, pH 7.2, to contain 5×10^9 viable organisms. The animals should show no obvious adverse effects and there must be no mortality.

If this safety test has been performed with good results on a representative seed lot or batch of the test vaccine, it does not have to be repeated routinely on other vaccine lots prepared from the same seed lot and using the same manufacturing process.

A safety test on *B. abortus* strain RB51 vaccine is not routinely done. If desired, 8–10-week-old female Balb/c mice can be injected intraperitoneally with 1×10^8 CFUs and the spleens cultured at 6 weeks post-inoculation. Spleens should be free from RB51 and the mice should not develop anti-OPS antibodies.

c) Potency

• **S19 vaccine**

An S19 vaccine is efficient if it possesses the characteristics of the S19 original strain, i.e. if it is satisfactory with respect to identity, smoothness, immunogenicity and residual virulence (7). Batches should also be checked for the number of viable organisms.

• **Identity**

The reconstituted S19 vaccine should not contain extraneous microorganisms. *Brucella abortus* present in the vaccine is identified by suitable morphological, serological and biochemical tests and by culture: *Brucella abortus* S19 has the normal properties of a biovar 1 strain of *B. abortus*, but does not require CO_2 for growth, does not grow in the presence of benzylpenicillin (3 µg/ml = 5 IU/ml), thionin blue (2 µg/ml), and i-erythritol (1 mg/ml) (all final concentrations).

• **Smoothness (determination of dissociation phase)**

The S19 vaccine reconstituted in distilled water is streaked across six agar plates (serum–dextrose agar or trypticase–soy agar (TSA) with added serum 5% [v/v] or yeast extract 0.1 % [w/v] in such a manner that the colonies will be close together in certain areas, while semi-separated and separated in others. Slight differences in appearance are more obvious in adjacent than widely separated colonies. Plates are incubated at 37°C for 5 days and examined by obliquely reflected light (Henry's method) before and after staining (three plates) with crystal violet (White & Wilson's staining method).

Appearance of colonies before staining: S colonies appear round, glistening and blue to blue-green in colour. R colonies have a dry, granular appearance and are dull yellowish-white in colour. Mucoid colonies (M) are transparent and greyish in colour and can be distinguished by their slimy consistency when touched with a loop. Intermediate colonies (I), which are the most difficult to classify, have an appearance intermediate between S and R forms: they are slightly opaque and more granular than S colonies.

Appearance of colonies after staining with crystal violet: S colonies do not take up the dye. Dissociated colonies (I, M, or R) are stained various shades of red and purple and the surface may show radial cracks. Sometimes a stained surface film slips off a dissociated colony and is seen adjacent to it.

The colony phase can be confirmed by the acriflavine agglutination test (1). S colonies remain in suspension, whereas R colonies are agglutinated immediately and, if mucoid, will form threads. Intermediate colonies may remain in suspension or a very fine agglutination may occur.

• **Enumeration of live bacteria**

Inoculate each of at least five plates of tryptose, serum–dextrose or other suitable agar medium with 0.1 ml of adequate dilutions of the vaccine spread with a sterile glass, wire or plastic spreader. CFU per vaccine volume unit are enumerated.

• **Residual virulence (50% persistence time or 50% recovery time) (7, 21, 33, 71)**

i) Prepare adequate suspensions of both the *B. abortus* S19 seed lot or batch to be tested (test vaccine) and the S19 original seed culture (as a reference strain). For this, harvest a 24–48 hours growth of each strain in sterile buffered saline solution (BSS: NaCl 8.5 g; KH_2PO_4 1.0 g; K_2HPO_4 2.0 g; distilled water 1000 ml; pH 6.8) and adjust the suspension in BSS to 10^9 CFU/ml using a spectrophotometer (0.170 OD when read at 600 nm). The exact number of CFU/ml should be checked afterwards by plating serial tenfold dilutions on to adequate culture medium (blood agar base or TSA are recommended).

ii) Inject subcutaneously 0.1 ml (10^8 CFU/mouse) of the suspension containing the test vaccine into each of 32 female CD1 mice, aged 5–6 weeks. Carry out, in parallel, a similar inoculation in another 32 mice using the suspension containing the S19 reference strain. The original seed S19 strain, which has been shown satisfactory with respect to immunogenicity and/or residual virulence, can be obtained from USDA (see footnote 2 for address).

iii) Kill the mice by cervical dislocation, in groups of eight selected at random 3, 6, 9 and 12 weeks later.

iv) Remove the spleens and homogenise individually and aseptically with a glass grinder (or in adequate sterile bags with the Stomacher) in 1 ml of sterile BSS.

v) Spread each whole spleen suspension *in toto* on to several plates containing a suitable culture medium and incubate in standard *Brucella* conditions for 5–7 days (lower limit of detection: 1 bacterium per spleen). An animal is considered infected when at least 1 CFU is isolated from the spleen.

vi) Calculate the 50% persistence time or 50% recovery time (RT_{50}) by the SAS® statistical method specifically developed for RT_{50} calculations (to obtain the specific SAS® file see footnote 5 for

address). For this, determine the number of cured mice (no colonies isolated in the spleen) at each slaughtering point time (eight mice per point) and calculate the percentage of cured accumulated mice over time, by the Reed and Muench method (described in ref. 5). The function of distribution of this percentage describes a sigmoid curve, which must be linearised for calculating the RT_{50} values, using the computerised PROBIT procedure of the SAS® statistical package.

vii) Compare statistically the parallelism (intercept and slope) between the distribution lines obtained for both tested and reference S19 strains using the SAS® file specifically designed for this purpose. Two RT_{50} values can be statistically compared exclusively when they come from parallel distribution lines. If parallelism does not exist, the residual virulence of the tested strain should be considered inadequate, and discarded for vaccine production.

viii) If the parallelism is confirmed, compare statistically the RT_{50} values obtained for both tested and reference S19 strains using a SAS® file specifically designed for this purpose. To be accepted for vaccine production, the RT_{50} obtained with the tested strain should not differ significantly from that obtained with the reference S19 strain (RT_{50} and confidence limits are usually around 7.0 ± 1.3 weeks).

The underlying basis of the statistical procedure for performing the above residual virulence calculations have been recently described in detail (5–7). Alternatively, the statistical calculations described in steps vi) to viii) can be avoided by an easy-to-use specific HTML-JAVA script program (Rev2) recently developed and available free at: http://www.afssa.fr/interne/Rev2.html (71).

If this test has been done with good results on a representative seed lot or batch of the test vaccine, it does not have to be repeated routinely on other vaccine lots prepared from the same seed lot and using the same manufacturing process.

- **Immunogenicity in mice (5, 6)**

This test uses three groups of six female CD1 mice, aged 5–7 weeks, that have been selected at random.

i) Prepare and adjust spectrophotometrically the vaccine suspensions as indicated above.

ii) Inject subcutaneously a suspension containing 10^5 CFU (in a volume of 0.1 ml/mouse) of the vaccine to be examined (test vaccine) into each of six mice of the first group.

iii) Inject subcutaneously a suspension containing 10^5 CFU of live bacteria of a reference S19 vaccine into each of six mice of the second group. The third group will serve as the unvaccinated control group and should be inoculated subcutaneously with 0.1 ml of BSS.

iv) The exact number of CFU inoculated should be checked afterwards by plating serial tenfold dilutions on to adequate culture medium (blood agar base or TSA are recommended).

v) All the mice are challenged 30 days after vaccination (and immediately following 16 hours' starvation), intraperitoneally with a suspension (0.1 ml/mouse) containing 2×10^5 CFU of *B. abortus* strain 544 (CO_2-dependent), prepared, adjusted and retrospectively checked as above.

vi) Kill the mice by cervical dislocation 15 days later.

vii) Each spleen is excised aseptically, the fat is removed, and the spleen is weighed and homogenised. Alternatively, the spleens can be frozen and kept at –20°C for from 24 hours to 7 weeks.

viii) Each spleen is homogenised aseptically with a glass grinder (or in adequate sterile bags in Stomacher) in nine times its weight of BSS, pH 6.8 and three serial tenfold dilutions (1/10, 1/100 and 1/1000) of each homogenate made in the same diluent. Spread 0.2 ml of each dilution by quadruplicate in agar plates and incubate two of the plates in a 10% CO_2 atmosphere (allows the growth of both vaccine and challenge strains) and the other two plates in air (inhibits the growth of the *B. abortus* 544 CO_2-dependent challenge strain), both at 37°C for 5 days.

ix) Colonies of *Brucella* should be enumerated on the dilutions corresponding to plates showing fewer than 300 CFU. When no colony is seen in the plates corresponding to the 1/10 dilution, the spleen is considered to be infected with five bacteria. These numbers of *Brucella* per spleen are first recorded as X and expressed as Y, after the following transformation: Y = log (X/log X). Mean and standard deviation, which are the response of each group of six mice, are then calculated.

x) The conditions of the control experiment are satisfactory when: i) the response of unvaccinated mice (mean of Y) is at least of 4.5; ii) the response of mice vaccinated with the reference S19 vaccine is lower than 2.5; and iii) the standard deviation calculated on each lot of six mice is lower than 0.8.

xi) Carry out the statistical comparisons (the least significant differences [LSD] test is recommended) of the immunogenicity values obtained in mice vaccinated with the S19 strain to be tested with respect to

those obtained in mice vaccinated with the reference vaccine and in the unvaccinated control group. The test vaccine would be satisfactory if the immunogenicity value obtained in mice vaccinated with this vaccine is significantly lower than that obtained in the unvaccinated controls and, moreover, does not differ significantly from that obtained in mice vaccinated with the reference vaccine. (For detailed information on this procedure, see footnote 5 for contact address.)

If this test has been done with good results on a representative batch of the test vaccine, it does not have to be repeated routinely on other vaccine lots prepared from the same seed lot and with the same manufacturing process.

- **RB51 vaccine**

  As dosage (CFU) of the master seed was correlated to protection as part of licensure of RB51 for cattle in the USA, *in vivo* potency tests are not routinely conducted for serials of the RB51 vaccine. In the USA, plate counts of viable organisms have been approved and used as a measure of potency (this approach is identical to the potency test for S19 vaccine in the USA). A test in Balb/c female mice using 1×10^4 *B. abortus* strain 2308 organisms as the challenge strain has been proposed, but the correlation of this test to vaccine protection in cattle has not been completely determined. In the USA plate counts of viable organisms have been approved and used (82). Rough vaccines for brucellosis have been discussed in some detail (53).

d) Duration of immunity

Vaccinating calves with a full dose of S19 vaccine is considered to give long-lasting immunity, and subsequent doses are not recommended. However, there is no proven evidence for this and revaccination could be advisable in endemic areas.

The duration of immunity induced by RB51 vaccine in cattle is unknown, whatever the dose applied and the age at vaccination.

e) Stability

Brucella abortus S19 vaccine prepared from seed stock from appropriate sources is stable in characteristics, provided that the in-process and batch control requirements described above are fulfilled, and shows no tendency to reversion to virulence. The lyophilised vaccine shows a gradual loss of viable count, but should retain its potency for the recommended shelf life. Allowance for this phenomenon is normally made by ensuring that the viable count immediately following lyophilisation is well in excess of the minimum requirement. Maintenance of a cold chain during distribution of the vaccine will ensure its viability.

Brucella abortus strain RB51 has shown no tendency to revert to virulent smooth organisms after many passages *in vitro* or *in vivo*. This is probably due to the nature and place of the mutations found in this strain. *Brucella abortus* strain RB51, among other unknown mutations, has its *wbo*A gene disrupted by an IS711 element impeding synthesis of OPS. Despite this, it has been reported that this strain accumulates low amounts of cytoplasmic M-like OPS (16).

f) Preservatives

Antimicrobial preservatives must not be used in live S19 or *B. abortus* strain RB51 vaccines. For preparation of the lyophilised vaccine, a stabiliser containing 2.5% casein digest, e.g. Tryptone (Oxoid), 5% surcose and 1% sodium glutamate, dissolved in distilled water and sterilised by filtration is recommended.

g) Precautions (hazards)

Brucella abortus S19 and RB51, although attenuated strains, are still capable of causing disease in humans. Accordingly cell cultures and suspensions must be handled under appropriate conditions of biohazard containment. Reconstitution and subsequent handling of the reconstituted vaccine should be done with care to avoid accidental injection or eye or skin contamination. Vaccine residues and injection equipment should be decontaminated with a suitable disinfectant (phenolic, iodophor or aldehyde formulation) at recommended concentration. Medical advice should be sought in the event of accidental exposure. The efficacy of the antibiotic treatment of infections caused by S19 and RB51 in humans has not been adequately established. If S19 contamination occurs, a combined treatment with doxycycline plus rifampicin could be recommended. In the case of contamination with RB51 (a rifampicin-resistant strain), the treatment with rifampicin should be avoided and a regimen of doxycycline plus streptomycin or gentamcycin should be used except in pregnant women, which should be treated with trimethoprim and sulfa-methoxazole.

5. Tests of the final product

a) Safety

See Section C2.4.b. If this safety test has been performed with good results on a representative seed lot or batch of the test vaccine, it does not have to be repeated routinely on other vaccine lots prepared from the same seed lot and using the same manufacturing process.

b) Potency

Potency can also be determined on the final lyophilised product. The procedure is as described above in Section C2.4.c. If this potency test has been performed with good results on a representative seed lot or batch of the test vaccine, it does not have to be repeated routinely on other vaccine lots prepared from the same seed lot and using the same manufacturing process.

REFERENCES

1. ALTON G.G., JONES L.M., ANGUS R.D. & VERGER J.M. (1988). Techniques for the Brucellosis Laboratory. Institut National de la Recherche Agronomique, Paris, France.

2. ANGUS R.D. & BARTON C.E. (1984). The production and evaluation of a buffered plate antigen for use in a presumptive test for brucellosis. *Dev. Biol. Stand.*, **56**, 349–356.

3. ASARTA A. (1989). Erradicación de la brucelosis en el ganado vacuno de Navarra. *In:* Actas del XII Congreso Nacional de Microbiología. Sociedad Española de Microbiología (ed.), SEM, Pamplona, 371–371.

4. ASHFORD D.A., DI PIETRA J., LINGAPPA J., WOODS C., NOLL H., NEVILLE B., WEYANT R., BRAGG S.L., SPIEGEL R.A., TAPPERRRO J. & PERKINS B.A. (2004). Adverse events in humans associated with accidental exposure to the livestock brucellosis vaccine RB51. *Vaccine,* **22**, 3435–3439.

5. BONET-MAURY P., JUDE A. & SERVANT P. (1954). La mesure statistique de la virulence et l'immunité. Application á l'etude de la virulence du bacille typhique et á la mesure du pouvoir immunisant des vaccins antityphoidiques. *Rev. d'Immun. Th. Antimic.*, **18**, 21–49.

6. BOSSERAY N. (1992). Le vaccin Rev.1: dérive des caractères d'immunogénicité et de virulence indépendante des marqueurs classiques. *In:* Prevention of Brucellosis in the Mediterranean Countries, Plommet M., ed. Pudoc Scientific, Wageningen, The Netherlands, 182–186.

7. BOSSERAY N. (1993). Control methods and thresholds of acceptability for anti-*Brucella* vaccines. *Dev. Biol. Stand.*, **79**, 121–128.

8. BRACEWELL C.D. & CORBEL M.J. (1980). An association between arthritis and persistent serological reactions to *B. abortus* in cattle from apparently brucellosis-free herds. *Vet. Rec.,* **106**, 99.

9. BRICKER B.J. (2002). PCR as a diagnostic tool for brucellosis, *Vet. Microbiol.,* **90**, 435–446.

10. BRICKER B.J., EWALT D.R. & HALLING S.M. (2003). Brucella 'HOOF-Prints' strain typing by multi-locus analysis of variable number tandem repeats (VNTRs). *BMC Microbiol.*, 11 June, **3**, 15.

11. BRICKER B.J., EWALT D.R., OLSEN S.C. & JENSEN A.E. (2003). Evaluation of the *Brucella abortus* species-specific polymerase chain reaction assay, an improved version of the *Brucella* AMOS polymerase chain reaction assay for cattle. *J. Vet. Diagn. Invest.*,**15**, 374–378.

12. BRICKER B.J. & HALLING S.M. (1994). Differentiation of *Brucella abortus* bv. 1, 2, and 4, *Brucella melitensis*, *Brucella ovis*, and *Brucella suis* bv. 1 by PCR. *J. Clin. Microbiol.,* **32**, 2660–2666.

13. BRICKER B.J. & HALLING S.M. (1995). Enhancement of the *Brucella* AMOS PCR assay for differentiation of *Brucella abortus* vaccine strains S19 and RB51. *J. Clin. Microbiol.,* **33**, 1640–1642.

14. CLOECKAERT A., GRAYON M. & GREPINET O. (2002). Identificiation of *Brucella melitensis* vaccine strain Rev.1 by PCR-RFLP based on a mutation in the rpsL gene, *Vaccine*, **7**, 19–20.

15. CLOECKAERT A., VERGER J.M., GRAYON M., PAQUET J.Y., GARIN-BASTUJI B., FOSTER G. & GODFROID J. (2001). Classification of *Brucella* spp. isolated from marine mammals by DNA polymorphism at the omp2 locus. *Microbes Infect.*, **3**, 29–38.

16. CLOECKAERT A., ZYGMUNT M. & GUILLOTEAU L. (2002). *Brucella abortus* vaccine strain RB51 produces low levels of M-lik O-antigen. *Vaccine*, **20**, 1820–1822.

17. CORBEL M.J., BRACEWELL C.D., THOMAS E.L. & GILL K.P.W. (1979). Techniques in the identification of *Brucella* species. *In:* Identification Methods for Microbiologists, Second Edition. Skinner F.A. & Lovelock D.W., eds. Academic Press, London, UK and New York, USA, 86–89.

18. CORBEL M.J. & HENDRY D.M.F.D. (1983). Methods for the identification of *Brucella*. Booklet 2085. Ministry of Agriculture, Fisheries and Food, Lion House, Alnwick, Northumberland, UK.

19. CORBEL M.J., STUART F.A., BREWER R.A., JEFFREY M. & BRADLEY R. (1989). Arthropathy associated with *Brucella abortus* Strain 19 vaccination in cattle. 1. Examination of field cases. *Br. Vet. J.,* **145**, 337.

20. DE MASSIS F., GIOVANNINI A., DI EMIDIO B., RONCHI G.F., TITTARELLI M., DI VENTURA M., NANNINI D.& CAPORALE V. (2005). Use of the complement fixation and brucellin skin tests to identify cattle vaccinated with *Brucella abortus* strain RB51. *Vet. Ital.*, **41** (4), 291–299.

21. DIAZ R., GARATEA P., JONES L.M. & MORIYON I. (1979). Radial immunodiffusion test with a *Brucella* polysaccharide antigen for differentiating infected from vaccinated cattle. *J. Clin. Microbiol.*, **10**, 37–41.

22. EWALT D.R. & BRICKER B.J. (2000). Validation of the abbreviated AMOS PCR as a rapid screening method for differentiation of *Brucella abortus* field strain isolates and the vaccine strains, 19 and RB51. *J. Clin. Microbiol.,* **38**, 3085–3086.

23. EWALT D.R. & BRICKER B.J. (2003). Identification and differentiation of *Brucella abortus* field and vaccine strains by BaSS-PCR. *In:* Methods in Molecular Biology, Volume 216: PCR Detection of Microbial Pathogens: Methods and Protocols, Saches K. & Frey J., eds. Humana Press, Totowa, NJ, USA, 97–108.

24. EWALT D.R., PAYEUR J.B., RHYAN J.C. & GEER P.L. (1997). *Brucella suis* biovar 1 in naturally infected cattle: a bacteriological, serological, and histological study. J*. Vet. Diagn. Invest.*, **10**, 417–420.

25. FARRELL I.D. (1974). The development of new selective medium for the Isolation of *Brucella abortus* from Contaminated Sources. *Res. Vet. Sci.,* **16**, 280–286.

26. FOSTER G., OSTERMAN B.S., GODFROID J., JACQUES I. & CLOECKAERT A. (2007). *Brucella ceti* sp. nov. and *Brucella pinnipedialis* sp. nov. for *Brucella* strains with cetaceans and seals as their preferred hosts. *Int. J. Syst. Evol. Microbiol.,* **57**, 2688–2693.

27. GALL D., NIELSEN K., FORBES L., COOK W., LECLAIR D., BALSEVICIUS S., KELLY L., SMITH P. & MALLORY M. (2001). Evaluation of the fluorescence polarization assay and comparison to other serological assays for detection of brucellosis in cervids. *J. Wildl. Dis.,* **37**, 110–118.

28. GALL D., NIELSEN K., FORBES L., DAVIS D., ELZER P., OLSEN S., BALSEVICIUS S., KELLY L., SMITH P., TAN S. & JOLY D. (2000). Validation of the fluorescence polarization assay and comparison to other serological tests for detection of serum antibody to *Brucella abortus* in bison. *J. Wildl. Dis.*, **36**, 469–476.

29. GARCIA-YOLDI D., MARIN C.M., De MIGUEL P.M., MUNOZ P.M., VIZMANOS J.L. & LOPEZ-GONI I. (2006) Multiplex PCR assay for the identification and differentiation of all *Brucella* species and the vaccine strains *Brucella abortus* S19 and RB51 and *Brucella melitensis* Rev1. *Clin. Chem.*, **52**, 779–781.

30. GARIN B., TRAP D. & GAUMONT R. (1985). Assessment of the EDTA seroagglutination test for the diagnosis of bovine brucellosis. *Vet. Rec.*, **117**, 444–445.

31. GODFROID J., SAEGERMAN C., WELLEMANS V., WALRAVENS K., LETESSON J.J., TIBOR A., MCMILLAN A., SPENCER S., SANAA M., BAKKER D., POUILLOT R. & GARIN-BASTUJI B. (2002). How to substantiate eradication of bovine brucellosis when aspecific serological reactions occur in the course of brucellosis testing. *Vet. Microbiol.*, **90**, 461–477.

32. GOPAUL K.K., KOYLASS M.S., SMITH C.J. & WHATMORE A.M. (2008). Rapid identification of *Brucella* isolates to the species level by real time PCR based single nucleotide polymorphism (SNP) analysis. *BMC Microbiology*, **8**, 86.

33. GRILLO M.J., BOSSERAY N. & BLASCO J.M. (2000). *In vitro* markers and biological activity in mice of seed lot strains and commercial *Brucella melitensis* Rev.1 and *Brucella abortus* B19 vaccines. *Biologicals*, **28**, 119–127.

34. HENDRY D.M.F.D., CORBEL M.J., BELL R.A. & STACK J.A. (1985). *Brucella* antigen production and standardisation. Booklet 2499. Ministry of Agriculture, Fisheries and Food, Lion House, Alnwick, Northumberland, UK.

35. HINIĆ V., BRODARD I., THOMANN A., CVETNIĆ Z., FREY J. & ABRIL C. (2008). Novel identification and differentiation of *Brucella melitensis*, *B. abortus*, *B. suis*, *B. ovis*, *B. canis*, and *B. neotomae* suitable for both conventional and real-time PCR systems. *J. Microbiol. Methods*, **75**, 375–378.

36. INTERNATIONAL AIR TRANSPORT ASSOCIATION (2006). Dangerous Goods Regulations, 44th Edition. International Air Transport Association, 800 Place Victoria, P.O. Box 113; Montreal, Quebec H4Z 1M1, Canada, 815 pp.

37. JENSEN A.E., CHEVILLE N.F., THOEN C.O., MACMILLAN A.P. & MILLER W.G (1999). Genomic fingerprinting and development of a dendrogram for *Brucella* spp. isolated from seals, porpoises, and dolphins. *J. Vet. Diagn. Invest.*, **11**, 152–157.

38. JIMENEZ DE BAGUES M.P., MARIN C. & BLASCO J.M. (1991). Effect of antibiotic therapy and strain 19 vaccination on the spread of *Brucella melitensis* within an infected dairy herd. *Prev. Vet. Med.*, **11**, 17–24.

39. JOINT FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS/WORLD HEALTH ORGANIZATION EXPERT COMMITTEE ON BRUCELLOSIS (1986). Technical Report Series 740, Sixth Report. WHO, Geneva, Switzerland.

40. JONES L.M., BERMAN D.T., MORENO E., DEYOE B.L., GILSDORF M.J., HUBER J.D. & NICOLETTI P.L. (1980). Evaluation of a radial immunodiffusion test with polysaccharide B antigen for diagnosis of bovine brucellosis. *J. Clin. Microbiol.*, **12**, 753–760.

41. KITTELBERGER R., REICHEL M., JOYCE M. & STAAK C. (1997). Serological crossreactivity between *Brucella abortus* and *Yersinia enterocolitica* O:9:III. Specificity of the *in vitro* antigen-specific gamma interferon test for bovine brucellosis diagnosis in experimentally *Yersinia enterocolitica* O:9 infected cattle. *Vet. Microbiol.*, **57**, 361–371.

42. LE FLÈCHE P., JACQUES I., GRAYON M., AL DAHOUK S., BOUCHON P., DENOEUD F., NÖCKLER K., NEUBAUER H., GUILLOTEAU L.A. & VERGNAUD G. (2006). Evaluation and selection of tandem repeat loci for a *Brucella* MLVA typing assay. *BMC Microbiol.*, **6**, 9.

43. LÓPEZ-GOÑI I., GARCÍA-YOLDI D., MARÍN C.M., DE MIGUEL M.J., MUÑOZ P.M., JACQUES I., GRAYON M., CLOECKAERT A., FERREIRA A.C., CARDOSO R., CORRÊA DE SÁ M.I., WALRAVENS K., ALBERT D. & GARIN-BASTUJI B. (2008). Evaluation of a multiplex PCR assay (Bruce-ladder) for molecular typing of all *Brucella* species and of the vaccine strains. *J. Clin. Microbiol.,* **46**, 3484–3487.

44. LORD V.R. & CHERWONOGRODZKY J.W. (1992). Evaluation of polysaccharide, lipopolysaccharide, and beta-glucan antigens in gel immunodiffusion tests for brucellosis in cattle. *Am. J. Vet. Res.*, **53**, 389–391.

45. LORD V.R., ROLO M.R. & CHERWONOGRODZKY J.W. (1989). Evaluation of humoral immunity to *Brucella* sp in cattle by use of an agar-gel immunodiffusion test containing a polysaccharide antigen. *Am. J. Vet. Res.*, **50**, 1813–1816.

46. MACMILLAN A.P. & COCKREM D.S. (1985). Reduction of non-specific reactions to the *Brucella abortus* serum agglutination test by the addition of EDTA. *Res. Vet. Sci.*, **38**, 288–291.

47. MACMILLAN A.P., GREISER-WILKE I., MOENNIG V. & MATHIAS L.A. (1990). A competition enzyme immunoassay for brucellosis diagnosis. *Dtsch Tierarztl. Wochenschr.,* **97**, 83–85.

48. MAINAR-JAIME R.C., MARÍN C.M., DE MIGUEL M.J., MUÑOZ P.M. & BLASCO J.M. (2008). Experiences on the use of RB51 vaccine in Spain. Proceedings of the Brucellosis 2008 International Conference, Royal Holloway College, University of London, UK, 10–13 September 2008, p. 40.

49. MARIN C.M., ALABART J.L. & BLASCO J.M. (1996). Effect of antibiotics contained in two *Brucella* selective media on growth of *B. abortus*, *B. melitensis* and *B. ovis*. *J. Clin. Microbiol.*, **34**, 426–428.

50. MICHAUX-CHARACHON S., BOURG G., JUMAS-BILAK E., GUIGUE-TALET P., ALLARDET-SERVENT A., O'CALLAHAN D. & RAMUZ M. (1997). Genome structure and phylogeny in genus *Brucella*. *J. Bacteriol.*, **179**, 3244–3249.

51. MORENO E., CLOECKAERT A. & MORIYON I. (2002). *Brucella* evolution and taxonomy. *Vet. Microbiol.*, **90**, 209–227.

52. MORGAN W.J.B., MACKINNON D.J., LAWSON J.R. & CULLEN G.A. (1969). The rose bengal plate agglutination test in the diagnosis of brucellosis. *Vet. Rec.,* **85**, 636–641.

53. MORIYON I. (2002). Rough vaccines in animal brucellosis. *In:* Proceedings of the CIHEAM Advanced Seminar – Human and Animal Brucellosis, Pamplona, Spain, 16–20 September 2002.

54. MORIYON I., GRILLO M.J, MONREAL D., GONZALEZ D., MARIN C.M., LOPEZ-GONI I., MAINAR-JAIME R.C., MORENO E. & BLASCO J.M. (2004). Rough vaccines in animal brucellosis: structural and genetic basis and present status. *Vet. Res.,* **35**, 1–38.

55. MUNOZ P., MARIN C., MONREAL D., GONZALES D., GARIN-BASTUJI B., DIAZ R., MAINAR-JAIME R., MORIYON I. & BLASCO J. (2005). Efficacy of several serological tests and antigens for the diagnosis of bovine brucellosis in the presence of false positive serological results due to *Yersinia enterocolitica* O:9. *Clin. Diagn. Lab. Immunol.,* **12**, 141–151.

56. NICOLETTI P. (1992). An evaluation of serologic tests used to diagnose brucellosis in buffaloes (*Bubalus bubalis*). *Trop. Anim. Health Prod.,* **24**, 40–44.

57. NIELSEN K. (2002). Diagnosis of brucellosis by serology. *Vet. Microbiol.,* **90**, 447–459.

58. NIELSEN K., GALL D., JOLLEY M., LEISHMAN G., BALSEVICIUS S., SMITH P., NICOLETTI P. & THOMAS F. (1996). A homogenous fluorescence polarisation assay for detection of antibody *to Brucella abortus. J. Immunol. Methods,* **195**, 161–168.

59. NIELSEN K., KELLY L., GALL D., BALSEVICIUS S., BOSSE J., NICOLETTI P. & KELLY W. (1996). Comparison of enzyme immunoassays for the diagnosis of bovine brucellosis. *Prev. Vet. Med.,* **26**, 17–32.

60. NIELSEN K., KELLY L., GALL D., NICOLETTI P. & KELLY W. (1995). Improved competitive enzyme immunoassay for the diagnosis of bovine brucellosis. *Vet. Immunol. Immunopathol.,* **46**, 285–291.

61. NIELSEN K., SAMAGH B.S., SPECKMANN G. & STEMSHORN B. (1979). The bovine immune response to *Brucella abortus.* II. Elimination of some sporadic serological reactions by chelation of divalent cations. *Can. J. Comp. Med.,* **43**, 420–425.

62. NIELSEN K., SMITH P., YU W., NICOLETTI P., ELZER P., ROBLES C., BERMUDEZ R., RENTERIA T., MORENO F., RUIZ A., MASSENGILL C., MUENKS Q., JURGERSEN G., TOLLERSRUD T., SAMARTINO L., CONDE S., FORBES L., PEREZ B., ROJAS X. & MINOS A. (2005). Towards a single screening test for brucellosis. *Rev. sci. tech. Off. int. Epiz.,* **24**, 1027–1038.

63. NIELSEN K., SMITH P., YU W., NICOLETTI P., ELZER P., VIGLIOCCO A., SILVA P., BERMUDEZ R., RENTERIA T., MORENO F., RUIZ A., MASSENGILL C., MUENKS Q., KENNY K., TOLLERSRUD T., SAMARTINO L., CONDE S., DRAGHI DE BENITEZ G., GALL D., PEREZ B. & ROJAS X. (2004). Enzyme immunoassay for the diagnosis of bovine brucellosis: chimeric protein A-protein G as a common enzyme labelled detection reagent for sera of different animal species *Vet. Microbiol.,* **101**, 123–129.

64. NIELSEN K., SMITH P., YU W., NICOLETTI P., JURGERSEN G., STACK J. & GODFROID J. (2006). Serological discrimination by indirect enzyme immunoassay between the antibody response to *Brucella* sp. and *Yersinia enterocolitica* O:9 in cattle and pigs. *Vet. Immunol. Immunopathol.,* **109**, 69–78.

65. OCAMPO-SOSA A.A., AGÜERO-BALBÍN J. & GARCÍA-LOBO J.M. (2005). Development of a new PCR assay to identify *Brucella abortus* biovars 5, 6 and 9 and the new subgroup 3b of biovar 3. *Vet. Microbiol.,* **110**, 41–51.

66. OLSEN S.C. (2000). Immune responses and efficacy after administration of commercial *Brucella abortus* strain RB51 vaccine to cattle. *Vet. Therapeutics,* **3**, 183–191.

67. OLSEN S.C. (2002). Responses of adult cattle to vaccination with a reduced dose of *Brucella abortus* Strain RB51. *Res. Vet. Sci.,* **59**, 135–140.

68. PALMER M, CHEVILLE N & JENSEN A (1996). Experimental infection of pregnant cattle with vaccine candidate *Brucella abortus* strain RB51: Pathologic, bacteriologic and serologic findings. *Vet. Pathol.,* **33**, 682–691.

69. PALMER M.V., OLSEN S.C. & CHEVILLE N.F. (1997). Safety and immunogenicity of *Brucella abortus* strain RB51 vaccine in pregnant cattle. *Am. J. Vet. Res.,* **58**, 472–477.

70. POUILLOT R., GARIN-BASTUJI B., GERBIER G., COCHE Y., CAU C., DUFOUR B. & MOUTOU F. (1997). The brucellin skin test as a tool to differentiate false positive serological reactions in bovine brucellosis. *Vet. Res.,* **28**, 365–374.

71. Pouillot R., Grillo M.J., Alabart J.L., Garin-Bastuji B. & Blasco J.M. (2004). Statistical procedures for calculating the residual virulence of *Brucella abortus* strain 19 (S19) and *Brucella melitensis* strain Rev.1 vaccines in mice: theoretical basis and practical applications. *Rev. sci. tech. Off. int. Epiz.*, **22** (3), 1051–1063.

72. Roop II, R.M., Preston-Moore D., Bagchi T. & Schurig G.G. (1987). Rapid Identification of smooth *Brucella* species with a monoclonal antibody. *J. Clin. Microbiol.*, **25**, 2090–2093.

73. Saergerman C., Vo T.-K.O., De Waele L., Gilson D., Bastin A., Dubray G., Flanagan P., Limet J.N., Letesson J.J. & Godfroid J. (1999). Diagnosis of bovine brucellosis by skin test: conditions for the test and evaluation of its performance. *Vet. Rec.,* **145**, 214–218.

74. Samartino L.E, Fort M., Gregoret R. & Schurig G.G. (2000) Use of B*rucella abortus* vaccine strain RB51 in pregnant cows after calfhood vaccination with strain 19 in Argentina. *Prev. Vet. Med.*, **45**, 193–199.

75. Sangari F.J., Garcia-Lobo J.M. & Aguero J. (1994). The *Brucella abortus* vaccine strain B19 carries a deletion in the erythritol catabolic genes*. FEMS Microbiol. Lett.,* **121**, 337–342.

76. Scholz H.C., Hubalek Z., Nesvadbova J., Tomaso H., Vergnaud G., Le Flèche P., Whatmore A.M., Al Dahouk S., Krüger M., Lodri C. & Pfeffer M. (2008). Isolation of *Brucella microti* from soil. *Emerg. Infect. Dis.*, **14** (8), 1316–1317.

77. Scholz H.C., Hubalek Z., Sedlacek I., Vergnaud G., Tomaso H., Al Dahouk S., Melzer F., Kämpfer P., Neubauer H., Cloeckaert A., Maquart M., Zygmunt M.S., Whatmore A.M., Falsen E., Bahn P., Göllner C., Pfeffer M., Huber B., Busse H-J. & Nöckler K. (2008). *Brucella microti* sp. nov., isolated from the common vole *Microtus arvalis. Int. J. Syst. Evol. Microbiol.,* **58** (2), 375–382.

78. Schurig G.G., Sriranganathan N. & Corbel M.J. (2002). Brucellosis vaccines: past, present and future, Veterinary microbiology, **90**, 479–496.

79. Scott J.C., Koylass M.S., Stubberfield M.R. & Whatmore A.M. (2007). Multiplex assay based on single-nucleotide polymorphisms for rapid identification of *Brucella* isolates at the species level. *Appl. Environ. Microbiol.,* **73** (22), 7331–7337.

80. Stack J.A., Perrett L.L., Brew S.D., MacMillan A.P. (1999). C-ELISA for bovine brucellosis suitable for testing poor quality samples. *Vet. Record,* **145**, 735–736.

81. Stevens M.G., Hennager S.G., Olsen S.C. & Cheville N.F. (1994). Serologic responses in diagnostic tests for brucellosis in cattle vaccinated with *Brucella abortus* 19 or RB51. *J. Clin. Microbiol.*, **32**, 1065–1066.

82. Stevens M.G., Olsen S.C., Pugh G.W. & Brees D. (1995). Comparison of immune responses and resistance to brucellosis in mice vaccinated with *Brucella abortus* 19 or RB51. *Infect. Immun.*, **63**, 264–270.

83. United States Department of Agriculture (USDA), Animal and Plant Health Inspection Services (APHIS) (2003). Availability of an Environmental Assessment for Licensing of *Brucella abortus* Vaccine, Strain RB-51, Live Culture. *Federal Register*, 18 Feb 2003, **68** (32), 7761.

84. Uzal F., Samartino L., Schurig G., Carrasco A., Nielsen K., Cabrera R. & Taddeo H. (2000). Effect of vaccination with *Brucella abortus* strain RB51 on heifers and pregnant cattle. *Vet. Res. Comm.*, **24**, 143–151.

85. Van Drimmelen G. & Horwell F. (1964). Preliminary findings with the use of *Brucella melitensis* strain Rev 1 as a vaccine against brucellosis in cattle. *OIE Bull.*, **62**, 987.

86. Vemulapalli R., McQuiston J.R., Schurig G.G., Sriranganathan N., Halling S.M. & Boyle S.M. (1999). Identification of an IS711 element interrupting the wboA gene of *Brucella abortus* vaccine strain RB51 and a PCR assay to distinguish strain RB51 from other *Brucella* species and strains. *Clin. Diagn. Lab. Immunol.,* **6**, 760–764.

87. Verger J.M. (1985). *B. melitensis* infection in cattle. *In: Brucella melitensis*, Plommet & Verger, eds. Martinus Nijhoff Publ., Dordrecht-Boston-Lancaster. 197–203.

88. Villarroel M., Grell M. & Saenz. R. (2000). Reporte de primer caso humano de aislamiento y tipificación de *Brucella abortus* RB51. *Arch. Med. Vet.,* **32**, 89–91.

89. Weynants V., Gilson D., Cloeckaert A., Tibor A., Denoel P.A., Godfroid F., Limet J.N. & Letesson J.J. (1997). Characterization of smooth-lipopolysaccharide and O polysaccharides of *Brucella* species by competition binding assays with monoclonal antibodies. *Infect. Immun.*, **65**, 1939–1943.

90. WEYNANTS V., GODFROID J., LIMBOURG B., SAEGERMAN C. & LETESSON J. (1995). Specific bovine brucellosis diagnosis based on in vitro specific gamma interferon production. *J. Clin. Microbiol.*, **33**, 706–712.

91. WEYNANTS V., WALRAVENS K., DIDEMBURGH C., FLANAGAN P., GODFROID J. & LETESSON J. (1998). Quantitative assessment by flow cytometry of T-lymphocytes producing antigen-specific gamma interferon in *Brucella* immune cattle. *Vet. Immunol. Immunopathol.*, **66**, 309–320.

92. WHATMORE A.M., PERRETT L.L. & MACMILLAN A.P. (2007). Characterisation of the genetic diversity of *Brucella* by multilocus sequencing. *BMC Microbiology*, **7**, 34.

93. WHATMORE A.M. SHANKSTER S.J., PERRETT L.L., MURPHY T.J., BREW S.D., THIRLWALL R.E. CUTLER S.J. & MACMILLAN A.P. (2006). Identification and characterization of variable-number tandem-repeat markers for typing of *Brucella* spp. *J. Clin. Microbiol.*, 44 (6), 1982–1993.

94. WORLD HEALTH ORGANIZATION (2004). WHO Laboratory Biosafety Manual, Third Edition. WHO, Geneva, Switzerland.

95. WORLD HEALTH ORGANIZATION (2005). Guidance on regulations for the Transport of Infectious Substances., WHO, Geneva, Switzerland, http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2005_22r%20.pdf

96. WRIGHT P.F., NILSSON E., VAN ROOIJ E.M.A., LELENTA M. & JEGGO M.H. (1993). Standardisation and validation of enzyme-linked immunosorbent assay techniques for the detection of antibody in infectious disease diagnosis. *Rev. sci. tech. Off. int. Epiz.,* **12**, 435–450.

97. WRIGHT P.F., TOUNKARA K., LELENTA M. & JEGGO M.H. (1997). International reference Standards: antibody standards for the indirect enzyme-linked immunosorbent assay. *Rev. Sci. Tech.,* **3**, 824–832.

*
* *

NB: There are OIE Reference Laboratories for Bovine brucellosis (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).

CHAPTER 2.7.2.

CAPRINE AND OVINE BRUCELLOSIS
(excluding *Brucella ovis*)

SUMMARY

Brucella melitensis (*biovars 1, 2 or 3*) is the main causative agent of caprine and ovine brucellosis. Sporadic cases caused by B. abortus *have been observed, but cases of natural infection are rare in sheep and goats.* Brucella melitensis *is endemic in the Mediterranean region, but infection is widespread world-wide. North America* (*except Mexico*) *is believed to be free from the agent, as are Northern and Central Europe, South-East Asia, Australia and New Zealand.*

Clinically, the disease is characterised by one or more of the following signs: abortion, retained placenta, orchitis, epididymitis and, rarely, arthritis, with excretion of the organisms in uterine discharges and in milk. Diagnosis depends on the isolation of Brucella *from abortion material, udder secretions or from tissues removed at post-mortem. Presumptive diagnosis of* Brucella *infection can be made by assessing specific cell-mediated or serological responses to* Brucella *antigens.*

Brucella melitensis *is highly pathogenic for humans, causing Malta fever, one of the most serious zoonoses in the world. All infected tissues, cultures and potentially contaminated materials should therefore be handled at containment level 3.*

Identification of the agent: Presumptive evidence of Brucella *is provided by the demonstration, by modified acid-fast staining of organisms typical of* Brucella *in abortion material or vaginal discharge, especially if supported by serological tests. The polymerase chain reaction* (*PCR*) *methods provide additional means of detection. Whenever possible,* Brucella *spp. should be isolated using selective or non-selective media by culture from uterine discharges, aborted fetuses, udder secretions or selected tissues, such as lymph nodes, spleen, uterus, testes and epididymes. Species and biovars should be identified by phage lysis, and by cultural, biochemical and serological criteria. Molecular methods have been developed that could also be used for complementary identification based on specific genomic sequences.*

Serological and allergic skin tests: The buffered Brucella *antigen tests* (*BBAT*) *and the complement fixation test* (*CFT*) *are usually recommended for screening flocks and individual animals. The serum agglutination test is not considered to be reliable for use in small ruminants. The indirect enzyme-linked immunosorbent assay* (*I-ELISA*) *and fluorescence polarisation assay* (*FPA*) *can also be used for screening purposes. For pooled samples, there are no useful tests such as the milk ring test for cattle. The brucellin allergic skin test can be used as a screening or complementary test in unvaccinated flocks, provided that a purified, lipopolysaccharide* (*LPS*)*-free, standardised antigen preparation is used. Results must then be interpreted in relation to the clinical signs, history, and results of serological or cultural examination.*

Requirements for vaccines and diagnostic biologicals: Brucella melitensis *strain Rev.1 remains the reference vaccine to immunise sheep and goats at risk of infection from* B. melitensis *and is the vaccine with which any other vaccines should be compared. Production of* Brucella *antigens or Rev.1 vaccine is based on a seed-lot system. Seed cultures to be used for antigens for serological and allergic skin tests and for vaccines should originate from reference centres. They must conform to minimal standards for viability, smoothness, residual infectivity and immunogenicity, purity, identity and safety, if applicable. Brucellin preparations for the intradermal test must be free of smooth lipopolysaccharide and must not produce nonspecific inflammatory reactions or interfere*

> *with serological tests. Antigens for* BBAT *and CFT must be prepared from smooth strains of*
> B. abortus*, strain 1119-3 or strain 99. Antigens for I-ELISA are prepared from* B. abortus *strain*
> *1119-3 or strain 99 or* B. melitensis *biovar 1 reference strain 16M or antigens prepared from*
> *different smooth* Brucella *strains. All antigens must comply with minimum standards for purity,*
> *sensitivity and specificity.*

A. INTRODUCTION

Brucellosis in sheep and goats (excluding *Brucella ovis* infection) is primarily caused by one of the three biovars of *B. melitensis*. Sporadic infections caused by *B. abortus* or *B. suis* have been observed in sheep and goats, but such cases are rare. Pathologically and epidemiologically, *B. melitensis* infection in sheep and goats is very similar to *B. abortus* infection in cattle (see Chapter 2.4.3 Bovine brucellosis). In most circumstances, the primary route of transmission of *Brucella* is the placenta, fetal fluids and vaginal discharges expelled by infected ewes and goats when they abort or have a full-term parturition. Shedding of *Brucella* is also common in udder secretions and semen, and *Brucella* may be isolated from various tissues, such as lymph nodes from the head, spleen and organs associated with reproduction (uterus, epididymides and testes), and from arthritic lesions (2).

Brucella melitensis infection in domestic and wild susceptible species (see Chapter 2.4.3) is not rare when these species are reared in close contact with sheep and goats in enzootic areas. The manifestations of brucellosis in these animals are similar to those in cattle or sheep and goats.

The World Health Organization (WHO) laboratory biosafety manual classifies *Brucella* (and particularly *B. melitensis*) in Risk group III. Brucellosis is readily transmissible to humans, causing acute febrile illness – undulant fever – which may progress to a more chronic form and can also produce serious complications affecting the musculo–skeletal, cardiovascular, and central nervous systems. Infection is often due to occupational exposure and is essentially acquired by the oral, respiratory, or conjunctival routes, but ingestion of dairy products constitutes the main risk to the general public. There is an occupational risk to veterinarians, abattoir workers and farmers who handle infected animals and aborted fetuses or placentas. Brucellosis is one of the most easily acquired laboratory infections, and strict safety precautions should be observed when handling cultures and heavily infected samples, such as products of abortion. Specific recommendations have been made for the safety precautions to be observed with *Brucella*-infected materials (for further details see Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities, and refs 1, 39, 94 and 95 of Chapter 2.4.3). Laboratory manipulation of live cultures or contaminated material from infected animals is hazardous, as is handling large volumes of *Brucella,* and must be done under containment level 3 or higher conditions, as outlined in Chapter 1.1.2, to minimise occupational exposure.

The classification, microbiological and serological properties of the genus *Brucella* and related species and biovars are given in the Chapter 2.4.3 Bovine brucellosis.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Refer to Chapter 2.4.3 Bovine brucellosis for the detailed agent identification procedure for *Brucella*.

2. Serological tests

In situations where bacteriological examination is not practicable, diagnosis of *Brucella* infection must often be based on serological methods (2, 21). In routine tests, anti-*Brucella* antibodies are detected in serum. The most widely used serum-testing procedures for the diagnosis of smooth *Brucella* infections in sheep and goats are the buffered *Brucella* antigen tests (BBAT), and the complement fixation test (CFT). The bulk milk ring test, which has been very useful in cattle, is ineffective in small ruminants.

In small ruminants, the BBAT and the CFT are the most widely used methods (20). The indirect enzyme-linked immunosorbent assay (I-ELISA) and the fluorescence polarisation assay (FPA) have shown similar diagnostic performance. All these tests are prescribed for international trade. The BBAT is not completely specific, but is adequate as a screening test for detecting infected flocks or for guaranteeing the absence of infection in brucellosis-free flocks. However, due to the relative lack of sensitivity of both tests, discrepancies between results obtained using the Rose Bengal test (RBT) and the CFT are not rare in infected sheep and goats (7). The results of the two tests should therefore be considered simultaneously to increase the likelihood of detecting infected individuals and to improve control of the disease in areas where it has not been completely eradicated (1, 5, 7). When, for practical or economic reasons, the CFT cannot be used simultaneously with the RBT in eradication programmes, it is recommended to improve the sensitivity of the RBT by using three volumes of serum and one

volume of antigen (e.g. 75 µl and 25 µl, respectively) in place of an equal volume of each. This simple modification increases RBT sensitivity and minimises the discrepancies between RBT and CFT results (7). Because antibodies induced after Rev.1 vaccination cannot be differentiated in both tests from those induced by *B. melitensis* infection, RBT and CFT results should be carefully interpreted according to the vaccination status in the flock. In addition, both tests are not specific enough to discriminate serological reactions due to *B. melitensis* from the false-positive reactions (FPSR) due to cross-reacting bacteria such as *Yersinia enterocolitica* O:9.

Good diagnostic results have been obtained in sheep and goats with indirect (I-) or competitive (C-) enzyme-linked immunosorbent assays (ELISAs) using various antigens, but generally the ELISAs that use antigens with a high content of smooth lipopolysaccharide (sLPS) are the most useful. The C-ELISA provides similar sensitivity to the classical tests, RBT and CFT, and the I-ELISA has greater sensitivity. Like these classical tests, both ELISAs are unable to differentiate *B.-melitensis*-infected animals from those recently vaccinated with the Rev.1 vaccine (22) or infected with cross-reacting bacteria. Some of these ELISAs have potential advantages in sensitivity and/or specificity with respect to both BBAT and CFT (17). Preliminary C-ELISAs studies with a periplasmic protein from *B. abortus* (27) or *B. melitensis* (12) as antigen have been applied in sheep and reported to be promising in differentiating Rev.1 vaccinated from *B. melitensis* infected animals (11, 14).

- **Reference sera**

The OIE reference standards are those against which all other standards are compared and calibrated. For the BBAT and CFT, please refer to chapter 2.4.3.Bovine brucellosis for antigen standardisation and test protocols. A caprine reference standard for ELISAs and FPA for sheep and goat antibodies has been developed and will be available to national reference laboratories soon[1].

- **Production of cells**

Please refer to Chapter 2.4.3 Bovine brucellosis. *Brucella abortus* biovar 1 strains 99 or 1119 are the only strains recommended for the preparation of BBAT and CFT in sheep and goats.

a) *Brucella*-**buffered antigen test (a prescribed test for international trade)**

Please refer to Chapter 2.4.3 Bovine brucellosis.

- **Antigen production**

Please refer to Chapter 2.4.3 Bovine brucellosis. Note that RB antigen made with *B. abortus* is usually used to test for *B. melitensis*. The standardisation of RB antigen, as it is prescribed in Chapter 2.4.3, provides a sufficient sensitivity to the BBAT for international trade purposes. Moreover, it helps assure an adequate specificity in free areas where FPSR occur because of cross-reacting bacteria such as *Yersinia enterocolitica* O:9. However this standardisation is probably the main cause of the reduced sensitivity of some RB antigen batches and of the discrepancies with the CFT (7). Therefore, when RBT is used in eradication programmes in endemic areas, it could be advisable to adjust the RB antigen titre so that it is positive at a 1/45 OIEISS dilution and negative at a 1/55 dilution, without affecting the specificity of the test. The discrepancies with the CFT can also be minimised by using three volumes of serum and one volume of antigen (e.g. 75 µl and 25 µl, respectively) in place of an equal volume of each as mentioned in the standard test procedure.

- **Test procedure**

Please refer to Chapter 2.4.3 Bovine brucellosis.

b) **Complement fixation test (a prescribed test for international trade)**

- **Antigen production**

Please refer to Chapter 2.4.3 Bovine brucellosis. Note that CF antigen made with *B. abortus* is used to test for *B. melitensis.*

- **Test procedure**

Please refer to Chapter 2.4.3 Bovine brucellosis.

---

1   Obtainable from the OIE Reference Laboratory for Brucellosis at Veterinary Laboratories Agency (VLA) Weybridge, New Haw, Addlestone, Surrey KT15 3NB, United Kingdom.

c)   Enzyme-linked immunosorbent assays (a prescribed test for international trade)

Several variations of the I-ELISA have been described using different antigen preparations, antiglobulin-enzyme conjugates, and substrate/chromogens. Several commercial I-ELISAs are available but before being used for international trade, their respective cut-off should have been properly established using the appropriate validation techniques (see Chapter 1.1.4 Principles of validation of diagnostic assays for infectious diseases) and these tests should be standardised against the above-mentioned Standard.

The test method is described in Chapter 2.4.3 Bovine brucellosis

d)   Fluorescence polarisation assay (a prescribed test for international trade)

The FPA for detection of caprine and ovine antibody to *Brucella* sp. is essentially the same as that described for cattle (for more details see Chapter 2.4.3); an example serum dilution used is 1/25 for the tube test and 1/10 for the plate test (23–26). It is a simple technique for measuring antigen/antibody interaction. The FPA may be used as a screening and/or confirmatory test. Before being used for international trade, the FPA cut-off should be properly established using the appropriate validation techniques (see Chapter 1.1.4 Principles of validation of diagnostic assays for infectious diseases) and the test should be standardised against the above-mentioned Standard.

3.   Other tests

a)   Brucellin skin test (an alternative test for international trade)

An alternative diagnostic test is the brucellin skin test, which can be used for screening unvaccinated flocks, provided that a purified (free of sLPS) and standardised antigen preparation (e.g. brucellin INRA) is used.

The brucellin skin test has a high sensitivity for the diagnosis of *B. melitensis* infection in small ruminants and, in absence of vaccination, is considered one of the most specific diagnostic tests (2, 5, 17, 20). This test is of particular value for the interpretation of FPSR due to infection with cross-reacting bacteria (FPSR affected animals are always negative in the skin test), especially in brucellosis-free areas.

Rev.1 vaccinated animals can react in this test for years (17). Therefore this test cannot be recommended either as the sole diagnostic test or for the purposes of international trade in areas where Rev.1 vaccine is used.

To obtain suitable results it is essential to use standardised brucellin preparations that do not contain sLPS, as this antigen may provoke antibody-mediated inflammatory reactions or induce antibodies that interfere with subsequent serological screening. One such preparation is brucellin INRA, which is prepared from a rough strain of *B. melitensis* that is commercially available[2].

- **Test procedure**

i)   A volume of 0.1 ml of brucellin is injected intradermally into the lower eyelid.

ii)   The test is read after 48 hours.

iii)   Any visible or palpable reaction of hypersensitivity, such as an oedematous reaction leading to an elevation of the skin or thickening of the eyelid (≥ 2 mm), should be interpreted as a positive reaction.

Although in the absence of vaccination the brucellin intradermal test is one of the most specific tests in brucellosis, diagnosis should not be made exclusively on the basis of positive intradermal reactions and should be supported by adequate serological tests. The intradermal inoculation of brucellin might induce a temporary anergy in the cellular immune response. Therefore an interval of 6 weeks is generally recommended between two tests repeated on the same animal.

b)   Native hapten tests

The native hapten-based gel precipitation tests[3] (as described in Chapter 2.4.3.) are also of interest in sheep and goats as they are very specific for discriminating the serological responses of infected animals (positive) from those induced in Rev.1 vaccinated animals (usually negative after a given time after vaccination).

---

2   Brucellergène OCB®, Synbiotics Europe, 2 rue Alexander Fleming, 69007 Lyon, France.
3   The detailed procedure could be obtained from the Departamento de Sanidad Animal, Centro de Investigacion y Tecnologia Agroalimentaria/Gobierno de Aragon, Avenida Montañana 930, 50059. Zaragoza. Spain.

The optimal diagnostic sensitivity (around 90%) is obtained in the double gel diffusion (DGD) or reverse radial immunodiffusion tests for sheep and goats, respectively (14, 20).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

C1. Brucellin

Please refer to Chapter 2.4.3 Bovine brucellosis.

C2. Vaccines

Brucella melitensis strain Rev.1 vaccine

The most widely used vaccine for the prevention of brucellosis in sheep and goats is the *Brucella melitensis* Rev.1 vaccine, which remains the reference vaccine with which any other vaccines should be compared. The RB51 vaccine is not effective in sheep against *B. melitensis* infection (16). In addition, other rough mutants defective in core and O-polysaccharide synthesis and export induce antibodies reacting in the I-ELISA with sLPS and are less effective than Rev.1 vaccine against *B. melitensis* infection in sheep (4). The Rev.1 vaccine is used as a freeze-dried suspension of live *B. melitensis* biovar 1 Rev.1 strain for the immunisation of sheep and goats. It should be normally given to lambs and kids aged between 3 and 6 months as a single subcutaneous or conjunctival inoculation. The standard dose is between 0.5×10^9 and 2.0×10^9 viable organisms. The subcutaneous vaccination induces strong interferences in serological tests and should not be recommended in combined eradication programmes (15, 22). However, when this vaccine is administered conjunctivally, it produces a similar protection without inducing a persistent antibody response, thus facilitating the application of eradication programmes combined with vaccination (15, 22). Care must be taken when using Rev.1 vaccine to avoid the risk of contaminating the environment or causing human infection. In many developing countries and endemic areas, vaccination of the whole population has to be considered as the best option for the control of the disease (6). However, Rev.1 vaccine is known to often cause abortion and excretion in milk when animals are vaccinated during pregnancy, either with a full or reduced dose (6). These side-effects are considerably reduced when adult animals are vaccinated conjunctivally (full dose) before mating or during the last month of pregnancy. Therefore, when mass vaccination is the only means of controlling the disease, a vaccination campaign should be recommended using the standard dose of Rev.1 administered by the conjunctival route when the animals are not pregnant or during the late lambing and prebreeding season (6).

The subcutaneous vaccination of young animals and the vaccination of adult animals, even at reduced doses, may lead to long-term persistence of vaccinal antibodies in a significant proportion of vaccinated animals that creates serious interferences in the serological diagnosis of brucellosis. As indicated above, conjunctival vaccination minimises these problems and thus it is the recommended method for combined eradication programmes. Therefore, the serological diagnosis of brucellosis should take into account the vaccinal state of the herd and the overall frequency distribution of antibody titres detected in the group of animals tested.

1. Seed management

a) Characteristics of the seed

Brucella melitensis biovar 1 strain Rev.1 original seed for vaccine production can be obtained commercially[4]. A European reference Rev.1 strain that possesses the characteristics of the Rev.1 original seed is also obtainable from the European Pharmacopoeia[5].

Production of *Brucella* live vaccines is based on the seed-lot system described above (Section B.2) for BBAT and CFT antigens. Strains should be cultured in a suitable medium. Strain Rev.1 must conform to the characteristics of *B. melitensis* biovar 1, except that it should grow more slowly. Additionally, when incubated in air (atmospheres containing CO_2 alter the results) at 37°C, it should grow on agar containing streptomycin (2.5 µg/ml), and it should be inhibited by the addition to a suitable culture medium of sodium benzylpenicillin (3 µg [5 International Units (IU)]/ml), thionin (20 µg/ml) or basic fuchsin (20 µg/ml). Recently, polymerase chain reaction and molecular techniques have been used to further characterise the vaccine (3, 13). It must also conform to the characteristics of residual virulence and immunogenicity in mice of the original seed.

---

4   Obtainable from the OIE Reference Laboratory for Brucellosis at AFSSA, 94706 Maisons-Alfort, France.
5   Obtainable from the European Pharmacopoeia, BP 907, 67029 Strasbourg Cedex 1, France.

b) Method of culture

Serum–dextrose agar, and trypticase–soy agar, to which 5% serum or 0.1% yeast extract may be added, are among the solid media that have been found to be satisfactory for propagating the Rev.1 strain (2, 28). Rev.1 strain does not grow well on potato agar.

For vaccine production, Rev.1 may be grown under conditions similar to those described for S99 and S1119-3 (see Chapter 2.4.3), except that Rev.1 generally needs 3–5 days to grow, the phenol saline is replaced by a freeze-drying stabiliser, and the organisms are not killed but are stored at 4°C while quality control examinations are carried out as described below. Moreover, the specific requirements for Rev.1 vaccine production recommend that: each seed lot (i.e. the culture used to inoculate medium for vaccine production) should be no more than three passages removed from an original seed culture and that the harvest of a vaccine lot should be no more than three passages from a seed lot or an original seed. The original seed culture should always be checked for the absence of dissociation before use. The recommended method for preparing seed material is given in ref. 2. The following freeze-drying stabiliser (sterilised by filtration) is of proven value: enzymatic digest of casein (2.5 g); sucrose (5 g); sodium glutamate (1 g); distilled water (100 ml).

c) Validation as a vaccine

Numerous independent studies have confirmed the value of *B. melitensis* strain Rev.1 as a vaccine for protecting sheep and goats from brucellosis. Its virulence is unchanged after passage through pregnant sheep and goats. Abortions may result when the Rev.1 vaccine is inoculated into pregnant ewes or goats. The vaccine-induced abortions are not avoided using reduced doses, and doses as low as 10^6, used either subcutaneously or conjunctivally, have been demonstrated to induce abortions and milk excretion of the vaccine strain (6).

A Rev.1 vaccine is efficient if it possesses the characteristics of the Rev.1 original strain, i.e. those of *B. melitensis* biovar 1 reference strain 16M (ATCC No. 23456), except those specific for the strain Rev.1 (2, 20), and if it proves to be satisfactory with respect to immunogenicity and residual virulence in the mouse model (9) (see below).

2. Method of manufacture (2, 28)

For production of *B. melitensis* strain Rev.1 vaccine, the procedures described above for antigens (2) can be used except that the cells are collected in a freeze-drying stabiliser and deposited by centrifugation. The yield from one fermenter run or the pooled cells from a batch of Roux flask cultures inoculated on the same occasion from the same seed lot constitutes a single harvest. More than one single harvest may be pooled to form the final bulk that is used to fill the final containers of a batch of vaccine. Before pooling, each single harvest must be checked for purity, cell concentration, dissociation and identity. The volume of the final bulk is adjusted by adding sufficient stabiliser so that a dose contains an appropriate number of viable organisms. After adjusting the cell concentration of the final bulk, tests for identity, dissociation and absence of contaminating organisms are conducted (see below).

3. In-process control

In-process checks should be made on the growth of Rev.1 vaccine from either solid or liquid medium to verify identity and to ensure purity and freedom from dissociation to rough forms during preparation of seed lots, single harvests, final bulks and the final (filling) lots. At least 99% of cells in seed lots and 95% of cells in final lots should be in the smooth phase.

Cell concentration should be estimated on the bulks and precisely determined on final lots. Immunogenicity and the residual virulence (50% persistence time or 50% recovery time) should also be determined on seed lots and final lots. If these tests have been done with good results on a representative batch of the test vaccine, it does not have to be repeated routinely on other vaccine lots prepared from the same seed lot and with the same manufacturing process.

4. Batch control

With freeze-dried vaccine, the control tests should be conducted on the vaccine reconstituted in the form in which it will be used.

a) Sterility (or absence of extraneous microorganisms)

The Rev.1 vaccine should be checked for bacterial and fungal contamination as prescribed in Chapter 1.1.9 Tests for sterility and freedom from contamination of biological materials.

b) Safety

The Rev.1 vaccine is a virulent product *per se*, and it should keep a minimal virulence to be efficacious (see Section C2.4.c in Chapter 2.4.3).

c) Potency

A Rev.1 vaccine is efficient if it possesses the characteristics of the Rev.1 original strain, i.e. if it is satisfactory with respect to immunogenicity, and residual virulence (10). Batches should also be checked for the number of viable organisms.

- **Identity**

  The reconstituted Rev.1 vaccine should not contain extraneous microorganisms. *Brucella melitensis* present in the vaccine is identified by suitable morphological, serological and biochemical tests and by culture: when incubated in air at 37°C, Rev.1 strain is inhibited by addition to the suitable culture medium of 3 µg (5 IU) per ml of sodium benzyl-penicillin, thionin (20 µg/ml) or basic fuchsin (20 µg/ml); the strain grows on agar containing 2.5 µg per ml of streptomycin.

- **Smoothness (determination of dissociation phase)**

  Please refer to Chapter 2.4.3 Bovine brucellosis.

  Sometimes slight and difficult to observe differences, can be seen in the size of Rev 1 colonies. The small colonies (1–1.2 mm in diameter) are typical for Rev.1, but larger Rev. 1 colonies can appear depending on the medium used, the amount of residual moisture in the incubator atmosphere, and the presence or absence of CO_2. The frequency of variation in colony size occurs normally at a ratio of 1 large to 10^3 small colonies. Both Rev.1 variants are of the S (smooth) type. To avoid an increase in this colony size variation along successive passages, it is important to always select small colonies for preparation of seed lots.

- **Enumeration of live bacteria**

  Please refer to Chapter 2.4.3 Bovine brucellosis.

- **Residual virulence (50% persistence time or 50% recovery time) (8, 18)**

  The same technical procedures indicated for 50% recovery time (RT_{50}) calculation of S19 vaccine (see Chapter 2.4.3) have to be applied for Rev.1, except that *B. abortus* S19 seed lot or batch to be tested (test vaccine) and the S19 original seed culture (used as a reference strain), respectively, are replaced by the corresponding *B. melitensis* Rev.1 seed lot or batch to be tested (test vaccine) and the *B. melitensis* Rev.1 original seed culture as the reference strain. For the reference original Rev.1 strain, RT_{50} and confidence limits are around 7.9 ± 1.2 weeks. A given Rev.1 vaccine seed lot or batch should keep similar residual virulence to be acceptable.

If this test has been done with good results on a representative batch of the test vaccine, it does not have to be repeated routinely on other vaccine lots prepared from the same seed lot and with the same manufacturing process.

- **Immunogenicity in mice**

  The same technical procedures indicated for immunogenicity calculation of S19 vaccine (see Chapter 2.4.3) have to be applied for Rev.1, except that *B. abortus* S19 seed lot or batch to be tested (test vaccine) and the *B. abortus* S19 original seed culture (used as a reference strain), respectively, are replaced by the corresponding *B. melitensis* Rev.1 seed lot or batch to be tested (test vaccine) and the *B. melitensis* Rev.1 original seed culture as the reference strain.

  Conditions of the control experiment are satisfactory when: i) the response in unvaccinated mice (mean of Y) is at least of 4.5; ii) the response in mice vaccinated with the reference Rev.1 vaccine is lower than 2.5; and iii) the standard deviation calculated on each lot of six mice is lower than 0.8.

If this test has been done with good results on a representative batch of the test vaccine, it does not have to be repeated routinely on other vaccine lots prepared from the same seed lot and with the same manufacturing process.

d) Duration of immunity

It is accepted that subcutaneous or conjunctival vaccination with standard doses of Rev.1 confers a solid and durable immunity in sheep and goats. However, growing field evidence shows that the immunity conferred declines with time, and revaccination could be advisable in endemic areas.

The use of reduced doses of Rev.1 produces a less efficient immunity, while side-effects, such as antibody responses or induction of abortion, are not fully avoided.

e) Stability

Strain Rev.1 vaccine prepared from seed stock from appropriate sources is stable in characteristics provided that the in-process and batch control requirements described above are fulfilled, and shows no tendency to reversion to virulence. The lyophilised vaccine shows a gradual loss of viable count, but should retain its potency for the recommended shelf life. Allowance for this phenomenon is normally made by ensuring that the viable count immediately following lyophilisation is well in excess of the minimum requirement. Maintenance of a cold chain during distribution of the vaccine will ensure its viability.

f) Preservatives

Antimicrobial preservatives must not be used in live Rev.1 vaccine. For preparation of the freeze-dried vaccine, a stabiliser as described in Section C2.4.f of Chapter 2.4.3 is recommended.

g) Precautions (hazards)

Please refer to chapter 2.4.3. Bovine brucellosis. *Brucella melitensis* Rev.1, although an attenuated strain, is still capable of causing disease in humans. Accordingly, cell cultures and suspensions must be handled under appropriate conditions of biohazard containment (see Chapter 1.1.2). Reconstitution and subsequent handling of the reconstituted vaccine should be done with care to avoid accidental injection or eye or skin contamination. Vaccine residues and injection equipment should be decontaminated with a suitable disinfectant. Medical advice should be sought in the event of accidental exposure. The efficacy of the antibiotic treatment of infections caused by Rev.1 (a streptomycin-resistant strain) in humans has not been adequately established but data in mice suggest that if Rev.1 contamination occurs, a combined treatment with doxycycline plus rifampicin or gentamycin could be recommended (19).

5. Tests on the final product

a) Safety

See Section C2.4.b of Chapter 2.4.3.

b) Potency

For the freeze-dried vaccine, the potency must be determined on the final product. The tests are as described in Section C2.4.c of Chapter 2.4.3.

In order to assess the vaccine efficiency, a representative sample of previously seronegative animals vaccinated with each new vaccine batch should be bled 15–20 days after vaccination and the serum samples submitted to BBAT. If adequate and independently of the vaccination route used, more than 80% of vaccinated animals should be BBAT positive.

REFERENCES

1. ALTON G.G. (1990). *Brucella melitensis. In:* Animal Brucellosis, Nielsen K.H. & Duncan J.R., eds. CRC Press, Boca Raton, Florida, USA, 383–409.

2. ALTON G.G., JONES L.M., ANGUS, R.D. & VERGER J.M. (1988). Techniques for the Brucellosis Laboratory. INRA, Paris, France.

3. BARDENSTEIN S., MANDELBOIM M., FICHT T.A., BAUM M. & BANAI M. (2002). Identification of the *Brucella melitensis* vaccine strain Rev.1 in animals and humans in Israel by PCR analysis of the PstI site polymorphism of its *omp*2 gene. *J. Clin. Microbiol.*, **40**, 1475–1480.

4. BARRIO M.B., GRILLÓ M.J., MUÑOZ P.M., JACQUES I., GONZALEZ D., DE MIGUEL M.J., MARÍN C.M., BARBERÁN M., LETESSON J.J., GORVEL J.P., MORIYÓN I., BLASCO J.M. & ZYGMUNT M.S. (2009). Rough mutants defective in core and O-polysaccharide synthesis and export induce antibodies reacting in an indirect ELISA with smooth lipopolysaccharide and are less effective than Rev 1 vaccine against *Brucella melitensis* infection of sheep. *Vaccine*, **27**,1741–1749.

5. BLASCO J.M. (1992). Diagnosis of *Brucella melitensis* infection in small ruminants. *In:* Prevention of Brucellosis in the Mediterranean Countries, Plommet M., ed. Pudoc Scientific, Wageningen, The Netherlands, 272–277.

6.   BLASCO J.M. (1997). A review on the use of *B. melitensis* Rev 1 vaccine in adult sheep and goats. *Prev. Vet. Med.*, **31**, 275–283.

7.   BLASCO J.M., GARIN-BASTUJI B., MARIN C.M., GERBIER G., FANLO J., JIMENEZ DE BAGUES M.P. & CAU C. (1994). Efficacy of different rose bengal and complement fixation antigens for the diagnosis of *Brucella melitensis* infection in sheep and goats. *Vet. Rec.,* **134**, 415–420.

8.   BOSSERAY N. (1991). *Brucella melitensis* Rev.1 attenuated vaccine: Stability of markers, residual virulence and immunogenicity in mice. *Biologicals*, **19**, 355–363.

9.   BOSSERAY N. (1992). Le vaccin Rev.1: dérive des caractères d'immunogénicité et de virulence indépendante des marqueurs classiques. *In:* Prevention of Brucellosis in the Mediterranean Countries, Plommet M., ed. Pudoc Scientific, Wageningen, The Netherlands, 182–186.

10. BOSSERAY N. (1993). Control methods and thresholds of acceptability for anti-*Brucella* vaccines. *Dev. Biol. Stand.,* **79**, 121–128.

11. CLOECKAERT A., BAUCHERON S., VIZCAINO N. & ZYGMUNT M.S. (2001). Use of recombinant BP26 protein in serological diagnosis of *Brucella melitensis* infection in sheep. *Clin. Diag Lab. Immunol.*, **8**, 772–775.

12. CLOECKAERT A., DEBBARH H.S., VIZCAINO N., SAMAN E., DUBRAY G. & ZYGMUNT M.S. (1996). Cloning, nucleotide sequence and expression of the *Brucella melitensis* bp26 gene coding for a protein immunogenic in infected sheep. *FEMS Microbiol. Lett.*, **140**, 139–144.

13. CLOECKAERT A., GRAYON M. & GRÉPIENT O. (2002). Identification of *Brucella melitensis* vaccine strain Rev.1 by PCR-RFLP based on a mutation in the *rpsL* gene. *Vaccine*, **20**, 2546–2550.

14. DEBBARH H.S., ZYGMUNT M.S., DUBRAY G. & CLOECKAERT A. (1996). Competitive ELISA using monoclonal antibodies to the *Brucella melitensis* bp26 protein to evaluate antibody response in infected and *B. melitensis* Rev1 vaccinated sheep. *Vet. Microbiol.*, **53**, 325–337.

15. DÍAZ-APARICIO E., MARÍN C., ALONSO B., ARAGÓN V., PÉREZ S., PARDO M., BLASCO J.M., DÍAZ R. & MORIYÓN I. (1994). Evaluation of serological tests for diagnosis of *Brucella melitensis* infection of goats. *J. Clin. Microbiol.*, **32**, 1159–1165.

16. EL-IDRISSI A.H., BENKIRANE A., EL-MAADOUDI M., BOUSLIKHANE M., BERRADA J. & ZEROUALI A. (2001). Comparison of the efficacy of *Brucella abortus* strain RB51 and *Brucella melitensis* Rev. 1 live vaccines against experimental infection with *Brucella melitensis* in pregnant ewes. *Rev. sci. tech. Off. int. Epiz.*, **20**, 741–747.

17. GARIN-BASTUJI B., BLASCO J.M., GRAYON M. & VERGER J.M. (1998). *Brucella melitensis* infection in sheep: present and future. *Vet. Res.*, 29, 255–274.

18. GRILLO M.J., BOSSERAY N. & BLASCO J.M. (2000). *In vitro* markers and biological activity in mice of seed lot strains and commercial *Brucella melitensis* Rev.1 and *Brucella abortus* B19 vaccines. *Biologicals*, **28**, 119–127.

19. GRILLO M.J., DE MIGUEL M.J., MUÑOZ P.M., MARIN C.M., ARIZA J. & BLASCO J.M. (2006). Efficacy of several antibiotic combinations against Brucella melitensis Rev 1 experimental infection in BALB/c mice *J. Antimicrob. Chemother.,* **58**, 622–626.

20. JOINT FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS/WORLD HEALTH ORGANIZATION EXPERT COMMITTEE ON BRUCELLOSIS (1986). Technical Report Series 740, Sixth Report. WHO, Geneva, Switzerland.

21. MACMILLAN A. (1990). Conventional serological tests. *In:* Animal Brucellosis, Nielsen K.H. & Duncan J.R., eds. CRC Press, Boca Raton, Florida, USA, 153–197.

22. MARIN C.M., MORENO E., MORIYON I., DIAZ R. & BLASCO J.M. (1999) Performance of competitive and indirect enzyme-linked immunosorbent assays, gel immunoprecipitation with native hapten polysaccharide, and standard serological tests in diagnosis sheep brucellosis. *Clin. Diagn. Lab. Immunol.,* **6**, 269–272.

23. NIELSEN K., GALL D., SMITH P., BALSEVICIUS S., GARRIDO F., DURAN FERRER M., BIANCIFIORI F., DAJER A., LUNA E., SAMARTINO L., BERMUDEZ R., MORENO F., RENTERIA T. & CORRAL A. (2004). Comparison of serological tests for detection of ovine and caprine antibody to *Brucella melitensis. Rev. sci. tech. Off. Int. Epiz.,* **23**, 979–987.

24. NIELSEN K., GALL D., SMITH P., BERMUDEZ R., MORENO F., RENTERIA T., RUIZ A., APARICIO L., VAZQUEZ A., DAJER A., LUNA E., SAMARTINO L. & HALBERT G. (2005). Evaluation of serological tests for the detection of caprine antibody to *Brucella melitensis. Small Rumin. Res.*, **56**, 253–258.

25. NIELSEN K., GALL D., SMITH P., VIGLIOCCO A., PERREZ B., SAMARTINO L., DAJER A., ELZER P. & ENRIGHT F. (1999). Validation of the fluorescence polarization assay as a serological test for the presumptive diagnosis of porcine brucellosis. *Vet. Microbiol.*, **68**, 245–253.

26. RAMIREZ-PFEIFFER C., NIELSEN K., MARIN-RICALDE F., RODRIGUES-PADILLA C., GOMEZ-FLORES R. (2006). Comparison of fluorescence polarization assay with card and complement fixation tests for the diagnosis of goat brucellosis in a high prevalence area. *Vet. Immunol. Immunopathol.,* **110**, 121–127.

27. ROSSETTI O., ARESE A., BOSCHIROLI L. & CRAVERO S. (1996). Cloning of *Brucella abortus* gene and characterisation of expressed 26 kilodalton periplasmic protein: potential use for diagnosis. *J. Clin. Microbiol.*, **34**, 165–169.

28. WORLD HEALTH ORGANIZATION (1977). Requirements for *Brucella melitensis* strain Rev.1 vaccine (Live – for veterinary use). World Health Organization (WHO) Technical Report Series No. 610, 28th Report, Annex 4. WHO, Geneva, Switzerland, 85–97*.*

*

* *

NB: There are OIE Reference Laboratories for Caprine and ovine brucellosis (excluding *Brucella ovis*) (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).

CHAPTER 2.7.9.

OVINE EPIDIDYMITIS
(*Brucella ovis*)

SUMMARY

Brucella ovis *produces a clinical or subclinical disease in sheep that is characterised by genital lesions in rams, and placentitis in ewes. Accordingly, the main consequences of the disease are reduced fertility in rams, infrequent abortions in ewes, and an increased perinatal mortality. The disease has been reported in Latin American, North American and European countries as well as Australia, New Zealand and South Africa, but probably occurs in most sheep-raising countries.*

Identification of the agent: *The existence of clinical lesions (unilateral or, occasionally, bilateral epididymitis) in rams may be indicative of the existence of infection, but laboratory examinations are necessary to confirm the disease. Laboratory confirmation may be based on direct or indirect methods. Direct diagnosis is made by means of bacteriological isolation of* B. ovis *from semen samples or tissues of rams, or vaginal discharges and milk of ewes, on adequate selective media. Molecular biological methods have being developed that could be used for complementary identification based on specific genomic sequences. The polymerase chain reaction (PCR) methods provide additional means of detection. However, indirect diagnosis based on serological tests is preferred for routine diagnosis.*

Serological tests: *The complement fixation test (CFT), agar gel immunodiffusion (AGID) test and indirect enzyme-linked immunosorbent assay (I-ELISA) using soluble surface antigens obtained from* B. ovis, *can be used. Some I-ELISAs using recombinant proteins and monoclonal antibodies are being tested in field trials. The sensitivities of the AGID test and ELISA are similar and sometimes the I-ELISA has higher sensitivity than the CFT. A combination of the AGID test and I-ELISA seems to give the best results in terms of sensitivity. However, with regard to simplicity and cost, the AGID test is the most practicable test for diagnosis of* B. ovis. *However, because of the lack of standardised methods recognised at the international level for I-ELISA and AGID, the prescribed test for international trade remains the CFT.*

Requirements for vaccines and diagnostic biologicals: *Seed cultures for antigen or vaccine production should be obtained from internationally recognised laboratories. A single standard dose (10^9 colony-forming units) of the live* B. melitensis *Rev.1 vaccine, administered subcutaneously or conjunctivally, can be used safely and effectively in rams, for the prevention of* B. ovis *infection. This vaccine strain should meet minimal quality standards: adequate concentration, absence of dissociation, adequate residual virulence and immunogenicity and free of extraneous agents (see Chapter 2.7.2 Caprine and ovine brucellosis [excluding* B. ovis]*).*

A. INTRODUCTION

Brucella ovis causes a genital infection of ovine livestock manifested by epididymitis, infrequent abortions, and increased lamb mortality. Passive venereal transmission via the ewe appears to be a frequent route of infection, but ram-to-ram transmission is also common[1] (2). Infected ewes may excrete *B. ovis* in vaginal discharges and

---

1   Under the semi-extensive production systems (most common in European Mediterranean countries) rams are usually housed together. Direct ram-to-ram transmission during non-breeding periods is quite frequent and has been suggested to take place by several routes, including the rectal mucosa. Most ram-to-ram infections, however, are produced through the oral route. Housed

milk and, accordingly, ewe-to-ram and lactating ewe-to-lamb transmission could also be determinant mechanisms of infection. Accordingly, the ewes are as relevant as rams in the epidemiology of infection, and control or eradication of *B. ovis* is feasible only if females are included in the corresponding programme.

The demonstration of the existence of genital lesions (unilateral or, occasionally, bilateral epididymitis) by palpating the testicles of rams may be indicative of the presence of this infection in a given flock. However, this clinical diagnosis is not sensitive enough because only about 50% of rams infected with *B. ovis* present epididymitis (2). Moreover, the clinical diagnosis is extremely nonspecific due to the existence of many other bacteria causing clinical epididymitis. The most frequently reported isolates causing epididymitis in rams include *Actinobacillus seminis, A. actinomycetemcomitans, Histophilus ovis, Haemophilus* spp., *Corynebacterium pseudotuberculosis ovis, B. melitensis* and *Chlamydophila abortus* (formerly *Chlamydia psittaci*) (4, 5, 8, 10, 12, 22, 27, 30). It must be emphasised that many palpable epididymal lesions in rams are sterile, trauma-induced spermatic granulomas.

Although cattle, goats and deer have been proved susceptible to *B. ovis* in artificial transmission experiments, natural cases have been reported only in deer (19). To date, no human cases have been reported, and *B. ovis* is considered to be non-zoonotic. However, in areas where *B. melitensis* infection co-exists with *B. ovis*, special care is required when handling samples, which should be transported to the laboratory in leak-proof containers (for further details see Chapter 2.4.3 Bovine brucellosis).

The classification, microbiological and serological properties of the genus *Brucella* and related species and biovars are given in the Chapter 2.4.3 Bovine brucellosis.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

a) Collection of samples

The most valuable samples for the isolation of *B. ovis* from live animals are semen, vaginal swabs and milk. For the collection of vaginal swabs and milk, see the instructions given in Chapter 2.7.2 Caprine and ovine brucellosis (excluding *B. ovis*). Semen (genital fluids) can be collected easily in swabs taken from the preputial cavity after electro-ejaculation. If an electro-ejaculator is not available, swabs can be taken from the vagina of brucellosis-free ewes immediately after natural mating.

For the isolation of *B. ovis* after necropsy, the preferred organs in terms of probability of isolation are the epididymides, seminal vesicles, ampullae, and inguinal lymph nodes in rams, and the uterus, iliac and supra-mammary lymph nodes in ewes. However, to obtain maximum sensitivity, a complete search that includes other organs and lymph nodes (spleen, cranial, scapular, prefemoral and testicular lymph nodes) should be performed. Dead lambs and placentas should also be examined. The preferred culture sites in aborted or stillborn lambs are abomasal content and lung.

Samples for culture should be refrigerated and transported to the laboratory to be cultured as soon as possible after collection. The organism remains viable for at least 72 hours at room temperature and survival is enhanced at 4°C or, preferably, by freezing the tissue samples.

b) Staining methods

Semen or vaginal smears can be examined following staining by Stamp's method (1, 7) (see Chapter 2.7.2), and characteristic coccobacilli should be demonstrated in many infected animals (28). Examination of Stamp-stained smears of suspect tissues (ram genital tract, inguinal lymph nodes, placentas, and abomasal content and lung of fetuses) may also allow a rapid presumptive diagnosis.

However, other bacteria with similar morphology or staining characteristics (*B. melitensis*, *Coxiella burnetii*, and *Chlamydophila abortus*) can also be present in such samples, making the diagnosis difficult for inexperienced personnel. Microscopy results should always be confirmed by culture of the microorganism.

---

rams establish hierarchies (head-to-head combats), and it is frequent that 'dominated' rams, after being 'mated' by the dominant rams, lick the prepuce of these dominant rams as an act of submission. If these dominant rams are infected, the probability of having *B. ovis* in the prepuce (excretion in the semen) is very high.

c)   Culture

The best direct method of diagnosis is bacteriological isolation on adequate culture media. Semen, vaginal swabs, or milk samples can be smeared directly on to plates with adequate culture media and incubated at 37°C in an atmosphere of 5–10% CO_2. Tissues should be macerated and ground in a small amount of sterile saline or phosphate buffered saline (PBS) with a stomacher or blender, before plating.

Growth normally appears after 3–4 days, but cultures should not be discarded as negative until 7 days have elapsed. Colonies of *B. ovis* become visible (0.5–2.5 mm) after 3–4 days of incubation, and are rough phase, round, shiny and convex.

Brucella ovis can be isolated in nonselective media, such as blood agar base enriched with 10% sterile ovine or bovine sera, or in blood agar medium with 5–10% sterile ovine blood. However, the inoculum frequently contains other bacteria, which often overgrow *B. ovis*. Accordingly, the use of selective media may be preferred. Various *B. ovis* selective media have been described. The modified Thayer–Martin's medium (3, 13) is recommended. Briefly, it can be prepared with GC medium base (38 g/litre; Biolife Laboratories, Milan, Italy) supplemented with haemoglobin (10 g/litre; Difco) and colistin methane-sulphonate (7.5 mg/litre), vancomycin (3 mg/litre), nitrofurantoin (10 mg/litre), nystatin (100,000 International Units [IU]/litre = 17.7 mg) and amphotericin B (2.5 mg/litre) (all products from Sigma Chemical, St Louis, United States of America [USA]). Working solutions are prepared as follows:

Solution A: Add 500 ml of distilled water to the GC medium base, heat the paste carefully while stirring continuously and autoclave at 120°C for 20 minutes.

Solution B: Suspend the haemoglobin in 500 ml of distilled water, adding the water slowly to avoid lumps. Once dissolved, add a magnetic stirrer and autoclave at 120°C for 20 minutes.

Antibiotic solution (prepared daily): colistin, nystatin and vancomycin are suspended in a mixture of methanol/water (1/1); nitrofurantoin is suspended in 1 ml of a 0.1 M NaOH sterile solution. For amphotericin B, it is recommended to prepare a stock solution of 10 mg/ml amphotericin B with 10 mg dissolved first in 1 ml sterile dimethyl sulphoxide (C_2H_6OS, for analysis; ACS) and then added to 9 ml of PBS (10 mM, pH 7.2). Any stock solution remaining can be stored some days at 4°C. All antibiotic solutions must be filtrated through 0.22 µm filters before addition to the culture medium.

Once autoclaved, stabilise the temperature (45–50°C) of both solutions A and B with continuous stirring. Mix both solutions (adding A to B), avoiding bubble formation. Add the antibiotic solutions while stirring continuously and carefully. Dispense into sterile plates.

Once prepared, the plates should not be stored for long periods, and freshly prepared medium is always recommended. This medium is also suitable for the isolation of *B. melitensis* (see Chapter 2.7.2).

All culture media should be subjected to quality control with the reference strain, to show that it supports growth.

Another suitable, but less effective, antibiotic combination is: vancomycin (3 mg/litre); colistin (7.5 mg/litre); nystatin (12,500 IU/litre); and nitrofurantoin (10 mg/litre).

The Farrell's medium described for the culture of smooth brucellae is not appropriate for the culture of *B. ovis* as it does not grow on this medium.

d)   Identification and typing

Brucella ovis colonies are not haemolytic. They are circular, convex, have unbroken edges, are always of the rough type when examined by oblique illumination, and test positive in the acriflavine test (1, 7). For growth, *B. ovis* needs an atmosphere of 5–10% CO_2. It lacks urease activity, fails to reduce nitrate to nitrite, is catalase positive and oxidase negative. It does not produce H_2S and, although it does not grow in the presence of methyl violet, it usually grows in the presence of standard concentrations of basic fuchsin and thionin. The cultures are not lysed by *Brucella*-phages of the Tbilissi (Tb), Weybridge (Wb) and Izatnagar (Iz) groups at the routine test dilution (RTD) or 10^4 RTD, while they are lysed by phage R/C (1, 7). Most laboratories are not equipped for a complete identification, and a practical schedule for presumptive identification is needed. Most *B. ovis* isolates can be correctly identified on the basis of growth characteristics, direct observation using obliquely reflected light, Gram or Stamp's staining, catalase, oxidase, urease and acriflavine tests. However, definitive identification should be carried out by reference laboratories with experience in identification and typing of *Brucella*.

The polymerase chain reaction (PCR) and other recently developed molecular methods provide additional means of detection and identification of *Brucella* sp. (see Chapter 2.4.3 Bovine brucellosis).

2. Serological tests

The most efficient and widely used tests are the complement fixation test (CFT), the double agar gel immunodiffusion (AGID) test and the indirect enzyme-linked immunosorbent assay (I-ELISA). Several countries have adopted various standard diagnostic techniques for *B. ovis*, but the only test prescribed by the OIE and the European Union (EU) for international trade is the CFT. However, it has been demonstrated that the AGID test shows similar sensitivity to the CFT, and it is a simpler test to perform. Although standardisation is lacking, numerous independent studies have shown that the I-ELISA is more sensitive and specific than either the CFT or AGID test, and with further validation and standardisation studies, the I-ELISA could become a suitable candidate for future designation as a prescribed test for *B. ovis* diagnosis.

The International Standard anti-*Brucella ovis* Serum (International Standard 1985[2]) is the one against which all other standards are compared and calibrated. This reference standard is available to national reference laboratories and should be used to establish secondary or national standards against which working standards can be prepared and used in the diagnostic laboratory for daily routine use.

- **Antigens**

    When rough *Brucella* cells are heat-extracted with saline (hot-saline method, HS), they yield water-soluble antigenic extracts, the major component of which precipitates with sera to rough *Brucella* (9, 18). For this reason, the HS extracts have been referred to as the 'rough-specific antigen' or, when obtained from *B. ovis*, as the *'B.-ovis-specific antigen'*. However, the chemical characterisation of the HS extracts from *B. ovis* has shown that they are enriched in rough lipopolysaccharide (R-LPS), group 3 outer membrane proteins and other outer membrane components (20). Thus, HS extracts contain LPS determinants specific for *B. ovis*, but also additional antigenic components, some of them shared with rough and smooth *B. melitensis* and other *Brucella* (23). Such components account for the cross-reactivity that is sometimes observed with the HS method and sera of sheep infected with *B. melitensis* or vaccinated with Rev.1 (20). The HS extract, due to its water solubility and high content of relevant cell-surface epitopes, is the best diagnostic antigen and has been widely used for the serological diagnosis of *B. ovis* infection.

    Brucella ovis REO 198, a CO_2- and serum-independent strain, is recommended as a source of the HS antigens to be used in serological tests[3]. Solid media described in Section B.1.c. are satisfactory for the growth of *B ovis* REO 198. HS antigen is prepared as follows:

    i)   Exponentially grow a suitable strain of *B ovis*, preferably aerobic and nonserum dependent, e.g. REO 198, in one of the following ways: for 48 hours in trypticase–soy broth flasks in an orbital incubator at 37°C and 150 rpm; or in Roux bottles of trypticase–soy agar, or other suitable medium, with 5% serum added (not necessary when using the REO 198 strain); or in a batch-type fermenter as described for *B. abortus,* but with the addition of 5% serum to the medium (not necessary when using the REO 198 strain).

    ii) Cells are resuspended in 0.85% saline or PBS, then washed twice in 0.85% saline (12 g of dried cells or 30 g of wet packed cells in 150 ml).

    iii) The cell suspension is then autoclaved at 120°C for 15–30 minutes.

    iv) After cooling, the suspension is centrifuged (15,000 *g*, 4°C, 15 minutes) and the supernatant fluid is filtered and dialysed against distilled water using 100 times the volume of the suspension, at 4°C; the water should be changed three times over a minimum of 2 days.

    v)   The dialysed fluid can be ultracentrifuged (100,000 *g*, 4°C, 6–8 hours), and the sediment is resuspended in a small amount of distilled water and freeze-dried. When produced to be used in the CFT, the addition of control process serum replacement II (CPSRII) prior to freeze-drying may assist in stability and anti-complementary activity.

    HS is then resuspended either in distilled water (for use in the AGID test), veronal buffered saline (for use in the CFT), or carbonate/bicarbonate buffer or PBS (for use in the I-ELISA) and titrated against a set of adequate positive and negative sera.

---

2   Obtainable from the OIE Reference Laboratory for Brucellosis at VLA Weybridge, Addlestone, Surrey KT15 3NB, United Kingdom.
3   Obtainable from the OIE Reference Laboratory for Brucellosis at AFSSA, 94706, Maisons-Alfort, France.

If it is to be used in the AGID test, the resuspended HS could be kept at 4°C adding 0.5% phenol as preservative. Freezing and thawing should be avoided (9). The CFT antigen should be standardised against the International anti-*B. ovis* Standard Serum to give 50% fixation at a 1/100 serum dilution.

a) **Complement fixation test (the prescribed test for international trade)**

There is no standardised method for the CFT, but the test is most conveniently carried out using the microtitration method. Some evidence shows that cold fixation is more sensitive than warm fixation (6, 21, 24), but that it is less specific. Anticomplementary reactions, common with sheep serum, are, however, more frequent with cold fixation.

Several methods have been proposed for the CFT using different concentrations of fresh sheep red blood cells (SRBCs) (a 2–3% suspension is usually recommended) sensitised with an equal volume of rabbit anti-SRBC serum diluted to contain several times (usually from two to five times) the minimum concentration required to produce 100% lysis of SRBCs in the presence of a titrated solution of guinea-pig complement. The latter is independently titrated (in the presence or absence of antigen according to the method) to determine the amount of complement required to produce either 50% or 100% lysis of sensitised SRBCs in a unit volume of a standardised suspension; these are defined as the 50% or 100% haemolytic unit of complement (C'H$_{50}$ or C'H$_{100}$), respectively. It is generally recommended to titrate the complement before each set of tests, a macromethod being preferred for an optimal determination of C'H$_{50}$. Usually, 1.25–2 C'H$_{100}$ or 5–6 C'H$_{50}$ are used in the test.

Barbital (veronal) buffered saline (VBS) is the standard diluent for the CFT. This is prepared from tablets available commercially, otherwise it may be prepared according to the formula described elsewhere (see Chapter 2.4.3 Bovine brucellosis). The test sera should be inactivated for 30 minutes in a water bath at 60–63°C, and then diluted (doubling dilutions) in VBS. The stock solution of HS antigen (2.5–20 mg/ml in VBS) is diluted in VBS as previously determined by titration (checkerboard titration). Usually, only one serum dilution is tested (generally 1/10).

Using standard 96-well microtitre plates with round (U) bottom, the technique is usually performed as follows:

i) Volumes of 25 µl of diluted inactivated test serum are placed in the well of the first and second rows. Volumes of 25 µl of CFT buffer are added to all wells except those of the first row. Serial doubling dilutions are then made by transferring 25 µl volumes of serum from the second row onwards.

ii) Volumes of 25 µl of antigen, diluted to working strength, are added to each well except wells in the first row.

iii) Volumes of 25 µl of complement, diluted to the number of units required, are added to each well.

iv) Control wells containing diluent only, complement + diluent, antigen + complement + diluent, are set up to contain 75 µl total volume in each case. A control serum that gives a minimum positive reaction should be tested in each set of tests to verify the sensitivity of test conditions.

v) The plates are incubated at 37°C for 30 minutes or at 4°C overnight, and a volume (25 or 50 µl according to the techniques) of sensitised SRBCs is added to each well. The plates are reincubated at 37°C for 30 minutes.

vi) The results are read after the plates have been centrifuged at 1000 ***g*** for 10 minutes at 4°C or left to stand at 4°C for 2–3 hours to allow unlysed cells to settle. The degree of haemolysis is compared with standards corresponding to 0, 25, 50, 75 and 100% lysis. The titre of the serum under test is the highest dilution in which there is 50% or less haemolysis.

• **Standardisation of the results of the complement fixation test**

There is a unit system that is based on the International Standard for anti-*Brucella ovis* Serum (International Standard 1985 [see footnote 2]). This serum contains 1000 IU/ml. If this serum is tested in a given method and gives a titre of, for example 100, then the factor for an unknown serum tested by that method can be found from the formula: 1000/100 ✕ titre of test serum = number of ICFTU (International CFT units) of antibody in the test serum per ml. It is recommended that any country using the CFT on a national scale should obtain agreement among the different laboratories performing the test by the same method, to allow the same level of sensitivity and specificity to be obtained against an adequate panel of sera from *B. ovis* culture positive and *Brucella*-free sheep. Results should always be expressed in ICFTU, calculated in relation to those obtained in a parallel titration with a standard serum, which itself may be calibrated against the International Standard.

Interpretation of the results: sera giving a titre equivalent to 50 ICFTU/ml or more are considered to be positive in the EU.

b) **Agar gel immunodiffusion test**

The AGID test (2) uses the following reagents: Good grade Noble agar or agarose, sodium chloride (NaCl), and borate buffer (prepared with boric acid [12.4 g]; potassium chloride [14.5 g]; distilled water [1600 ml]; adjusted to pH 8.3 with 0.2 M NaOH solution and made up to 2000 ml with distilled water).

To prepare the gels, dissolve 1 g of agarose (or Noble agar), 10 g of NaCl and 100 ml of borate buffer by boiling while stirring continuously. On a flat surface, cover clean glass slides with the necessary amount of molten gel to form a bed of 2.5 mm depth (3.5 ml approximately for standard microslides). After the gel has solidified (15–20 minutes), wells are cut in it using a gel puncher. The wells should be 3 mm in diameter and 3 mm apart, and should be arranged in a hexagonal pattern around a central well that is also 3 mm in diameter. The test can be adapted to Petri dishes and other patterns.

Sera to be examined are placed in alternate wells separated by a control positive serum (infection proved by bacteriology), with the antigen at its optimum concentration in the central well. The results are read after incubation for 24 and 48 hours at room temperature in a humid chamber. A positive reaction is a clearly defined precipitin line between the central well and the wells of the test sera that gives total or partial identity with that of the positive controls. Precipitin lines not giving total identity may also appear and correspond to minor antigenic components of HS extracts (antibodies to these components can also be common in infections due to *B. melitensis*). These reactions should also be considered to be positive. Before a definitive reading, it is important to wash the slides for 1 hour in a 5% sodium citrate water solution to clean unspecific precipitin lines.

The HS (2.5–20 mg/ml) diluted in distilled water (optionally containing 0.5% phenol as a preservative) is the antigen used in the AGID test (the preserved antigen can be stored at 4°C for at least 1 month). Dilutions of antigen are tested with a panel of 20–30 sera from rams naturally infected with *B. ovis* and with a panel of *Brucella*-free sheep. The optimum concentration of antigen is that giving the clearest precipitation line with all the sera from *B.-ovis*-infected rams being negative with the sera from *Brucella*-free sheep.

c)   Enzyme-linked immunosorbent assay (the alternative test for international trade)

Several variations of this assay have been proposed. The assay described here is an indirect I-ELISA using ABTS (2,2'-azino-bis-[3-ethylbenzothiazoline-6-sulphonic acid]) as chromogen, but other procedures are also suitable. Tests are performed on 96-well flat-bottomed ELISA plates. Reagent and serum dilutions are made in PBS, pH 7.2, with the addition of 0.05% Tween 20 (PBST). Antigen dilutions are made in a carbonate/bicarbonate buffer, pH 9.6, or, alternatively, in PBS, pH 7.2. Plates are washed after antigen coating and between incubations, where appropriate, usually with PBST. The antigen (HS) and conjugate are checkerboard titrated, and dilutions are selected to give the best discriminating ratio between negative and positive standard sera. Secondary antibodies (anti-ovine IgG [H + L chains]) antibodies are usually conjugated to horseradish peroxidase (HRPO), although other enzymes or conjugates (such as recombinant protein G/HRPO) can be used. A monoclonal antibody to bovine IgG_1–HRPO conjugate has also been found to be suitable for use in the I-ELISA (26). If a peroxidase conjugate is used, the chromogen, usually ABTS, is diluted in a substrate buffer (composed of citric acid trisodium and citric acid; pH 4). The substrate, hydrogen peroxide (H_2O_2), is added to this, and the plates are incubated for 15–25 minutes at room temperature. The reaction may be stopped with 1 mM sodium azide, and the colour change is read at 405–414 nm (for further details see Chapter 2.4.3).

The antigen used in the I-ELISA is the HS in stock solution at 1 mg/ml in coating buffer, titrated in a checkerboard titration, with different dilutions of antigen, conjugate and substrate, against a standard serum or against serial dilutions of a panel of sera from *B. ovis* culture positive and *Brucella*-free sheep to determine the most sensitive and specific dilution (usually 5–10 µg/ml).

- **Test procedure (example)**

i)   Microtitre plates of good quality polystyrene are coated by the addition of 100 µl of a predetermined antigen dilution in carbonate buffer, pH 9.6, to each well. Plates are incubated for 2 hours at 37°C. Alternatively, the coating can be made overnight at 4°C with 100 µl/well of the predetermined antigen dilution in PBS, pH 7.2. Plates are then washed four times to remove unbound antigen and dried by tapping firmly upside down on an absorbent paper. The coated plates can be used immediately or dried and stored at 4°C (the stability in these conditions is adequate for at least 1 month).

ii)   *Sera:* Dilute test and positive and negative control serum samples 1/200 by the addition of a minimum of 10 µl of serum to 2 ml PBST. This serum dilution is usually the optimal when using both polyclonal and monoclonal conjugates. However, dilutions of 1/50 are the optimal when using the protein G-peroxidase conjugate (14) Add 100 µl/well volumes of samples in duplicate to the microtitre plates. The plates are covered, incubated at 37°C for 1 hour, and washed three times with the PBST washing buffer.

iii) *Conjugate:* The titrated conjugate is diluted in PBST, added (100 µl) to the wells, and the plate is covered and incubated for 1 hour at 37°C. After incubation, the plates are washed again three times with PBST.

iv) *Substrate:* The solution of ABTS in substrate buffer is added (100 µl/well) and the plates are incubated for 15–60 minutes at room temperature with continuous shaking.

v) *Reading and interpreting the results:* Absorbance is read automatically in a spectrophotometer at 405–414 nm. Absorbance values may be expressed as percentages of the mean absorbance of the positive control or, preferably, transformed into I-ELISA units calculated either manually or by using a computer and a curve-fitting program from a standard curve constructed with the series of positive control dilution results.

The cut-off threshold should be properly established using the appropriate validation techniques (see Chapter 1.1.4 Principles of validation of diagnostic assays for infectious diseases). The International Standard for anti-*Brucella ovis* Serum or the corresponding secondary or national standards should be used to check or calibrate the particular test method in question.

Comparative studies have shown that the I-ELISA has better sensitivity than either the AGID test or the CFT (16, 21, 25, 31, 32). Due to the existence of some I-ELISA-negative and AGID-positive sera, the combination of the AGID test and I-ELISA gives optimal sensitivity (16). However, the combination of CF test and I-ELISA or CF and AGID tests does not improve the sensitivity of I-ELISA alone (16). Moreover, the CFT has other important disadvantages such as complexity, obligatory serum inactivation, anticomplementary activity of some sera, the difficulty of performing it with haemolysed sera, and prozone phenomena. Because of its sensitivity, simplicity and easy interpretation, the AGID test is very practicable for routine diagnosis in nonspecialised laboratories.

Little is known about the existence of false positive results in *B. ovis* serological tests as a consequence of infections due to bacteria showing cross-reacting epitopes with *B. ovis*. The foot rot agent (*Dichelobacter nodosus*) has been described as showing cross-reactions with *B. ovis* (29), but the extent and practical consequences of this cross-reactivity in *B. ovis* diagnostic tests is unknown[4].

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICAIS

Vaccination of both rams and ewes is probably the most economical and practical means for medium-term control of *B. ovis* in areas with a high incidence of infection. For long-term control, consideration should be given to the effect of vaccination on serological testing, and *B.-ovis*-free accreditation programmes have to be implemented. The live *B. melitensis* strain Rev.1 (see Chapter 2.7.2) is probably the best available vaccine for the prophylaxis of *B. ovis* infection (2). A single standard dose (10^9 colony-forming units) of Rev.1 administered subcutaneously (in a 1 ml volume) or conjunctivally (in a 25–30 µl volume), to 3–5 month-old animals confers adequate immunity against *B. ovis*. Conjunctival vaccination has the advantage of minimising the intense and long-lasting serological response evoked by subcutaneous vaccination, thereby improving the specificity of serological tests (2). When used in both young and adult males, the safety of the Rev.1 vaccine is adequate and side-effects appear to be very rare (15, 17). Therefore, in countries with extensive management and high levels of incidence, it would be advisable to vaccinate both young and healthy adult animals. In countries affected by *B. ovis* but free of *B. melitensis,* before using the *B. melitensis* Rev.1 vaccine account should be taken of possible serological interferences and the conjunctival route should be preferred to minimise this problem. The *B. abortus* RB51 live vaccine has not proven successful against *B. ovis* in sheep (11) and no alternative vaccines are currently available.

REFERENCES

1. ALTON G.G., JONES L.M., ANGUS R.D. & VERGER J.M. (1988). Techniques for the Brucellosis Laboratory. INRA, Paris, France.

2. BLASCO J.M. (1990). *Brucella ovis. In:* Animal Brucellosis, Nielsen K. & Duncan J.R., eds. CRC Press, Boca Raton, Florida, USA, 351–378.

---

4 *Arcanobacterium pyogenes* and *Corynebacterium ovis*, whose soluble extracts cross-react with *B. ovis* positive control sera, have been recently isolated from several lymph nodes of rams giving strong positive responses in *B. ovis* AGID test and I-ELISA (J.M. Blasco, unpublished results).

3.	BROWN G.M., RANGER C.R. & KELLEY D.J. (1971). Selective media for the isolation of *Brucella ovis. Cornell Vet.,* **61**, 265–280.

4.	BULGIN M.S. & ANDERSON B.C. (1983). Association of sexual experience with isolation of various bacteria in cases of ovine epididymitis. *J. Am. Vet. Med. Assoc.,* **182**, 372–374.

5.	BURGESS G.W. & McDOWELL J.W. (1981). *Escherichia coli* epididymitis and seminal vesiculitis in a ram. *Aust. Vet. J.,* **57**, 479–480.

6.	BURGESS G.W. & NORRIS M.J. (1982). Evaluation of the cold complement fixation test for diagnosis of ovine brucellosis. *Aust. Vet. J.,* **59**, 23–25.

7.	CORBEL M.J., GILL K.P.W. & THOMAS E.L. (1978). Methods for the identification of *Brucella.* Ministry of Agriculture, Fisheries and Food, UK, ADAS, RCV 22.

8.	DE LONG W.J., WALDHALM D.G. & HALL R.F. (1979). Bacterial isolates associated with epididymitis in rams from Idaho and eastern Oregon flocks. *Am. J. Vet. Res.,* **40,** 101–102.

9.	DIAZ R. & BOSSERAY N. (1973). Identification d'un composé antigénique spécifique de la phase rugueuse (R) des *Brucella. Ann. Rech. Vet.,* **4,** 283–292.

10.	EKDAHL M.O., MONEY D.F. & MARTIN C.A. (1968). Some aspects of epididymitis of rams in New Zealand. *N. Z. Vet. J.,* **16**, 81–82.

11.	JIMENEZ DE BAGUES M.P., BARBERAN M., MARIN C.M. & BLASCO J.M. (1995). The *Brucella abortus* RB51 vaccine does not confer protection against *Brucella ovis* in rams. *Vaccine*, **13**, 301–304.

12.	LIVINGSTONE C.W. & HARDY W.T. (1964). Isolation of *Actinobacillus seminis* from ovine epididymitis. *Am. J. Vet. Res.,* **25**, 660–663.

13.	MARIN C.M., ALABART J.L. & BLASCO J.M. (1996). Effect of antibiotics contained in two *Brucella* selective media on growth of *B. abortus, B. melitensis* and *B. ovis. J. Clin. Microbiol.*, **34**, 426–428.

14.	MARIN C.M., ALONSO-URMENETA B., MORIYON I., PEREZ S. & BLASCO J.M. (1998). Comparison of polyclonal, monoclonal and protein G peroxidase conjugates in an enzyme-linked immunosorbent assay for the diagnosis of *Brucella ovis* in sheep. *Vet. Rec.,* **143**, 390–394.

15.	MARIN C.M., BARBERAN M., JIMENEZ DE BAGUES M.P. & BLASCO J.M. (1990). Comparison of subcutaneous and conjunctival routes of Rev. 1 vaccination for the prophylaxis of *Brucella ovis* infection in rams. *Res. Vet. Sci.,* **48**, 209–215.

16.	MARIN C.M., JIMENEZ DE BAGUES M.P., BLASCO J.M., GAMAZO C., MORIYON I. & DIAZ R. (1989). Comparison of three serological tests for *Brucella ovis* infection of rams using different antigenic extracts. *Vet. Rec.,* **125**, 504–508.

17.	MUÑOZ P., DE MIGUEL M.J., GRILLÓ M.J., MARÍN C.M., BARBERÁN M. & BLASCO J.M. (2008). Immunopathological responses and kinetics of *B. melitensis* Rev 1 infection after subcutaneous or conjunctival vaccination in rams. *Vaccine*, **26**, 2562–2569.

18.	MYERS D.M., JONES L.M. & VARELA-DIAZ V. (1972). Studies of antigens for complement fixation and gel diffusion tests in the diagnosis of infections caused by *Brucella ovis* and other *Brucella. Appl. Microbiol.,* **23**, 894–902.

19.	RIDLER A. (2001) *Brucella ovis* infection in deer. *Surveillance*, **28** (3), 6–8.

20.	RIEZU-BOJ J.I., MORIYON I., BLASCO J.M., MARIN C.M. & DIAZ R. (1986). Comparison of lipopolysaccharide and outer membrane protein-lipopolysaccharide extracts in an enzyme-linked immunosorbent assay for the diagnosis of *Brucella ovis* infection. *J. Clin. Microbiol.,* **23**, 938–942.

21.	RIS D.R., HAMEL K.L. & LONG D.L. (1984). Comparison of an enzyme-linked immunospecific assay (ELISA) with the cold complement fixation test for the serodiagnosis of *Brucella ovis* infection. *N. Z. Vet. J.,* **32**, 18–20.

22   RODOLAKIS A. & BERNARD K. (1977). Isolement de *Chlamydia* des organes génitaux de béliers atteints d'épididymite. *Bull. Acad. Vet. Fr.,* **50**, 65–70.

23.   SANTOS J.M., VERSTREATE D.R., PERERA V.Y. & WINTER A.J. (1984). Outer membrane proteins from rough strains of four *Brucella* species. *Infect. Immun.,* **46**, 188–194.

24.   SEARSON J.E. (1982). Sensitivity and specificity of two microtitre complement fixation tests for the diagnosis of *Brucella ovis* infection in rams. *Aust. Vet. J.,* **58**, 5–7.

25.   SPENCER T.L. & BURGESS G.W. (1984). Enzyme-linked immunosorbent assay for *Brucella ovis* specific antibody in ram sera. *Res. Vet. Sci.,* **36**, 194–198.

26.   VIGLIOCCO A.M., SILVA PAULO P.S., MESTRE J., BRIONES G.C., DRAGHI G., TOSSI M. & NIELSEN K. (1997). Development and validation of an indirect enzyme immunoassay for detection of ovine antibody to *Brucella ovis.* *Vet. Microbiol.*, **54**, 357–368.

27.   WEBB R.F. (1983). Clinical findings and pathological changes in *Histophilus ovis* infections of sheep. *Res. Vet. Sci.,* **35**, 30–34.

28.   WEBB R.F., QUINN C.A., COCKRAM F.A. & HUSBAND A.J. (1980). Evaluation of procedures for the diagnosis of *Brucella ovis* infection in rams. *Aust. Vet. J.,* **56**, 172–175.

29.   WHITINGTON R.J., SAUNDERS V.F. & EGERTON J.R. (1996). Antigenic cross-reactions between the causative agent of ovine footrot, *Dichelobacter nodosus*, and other bacteria. *Small Rumin. Res.,* **22**, 55–67.

30.   WILLIAMSON P. & NAIRN M.E. (1980). Lesions caused by *Corynebacterium pseudotuberculosis* in the scrotum of rams. *Aust. Vet. J.,* **56**, 496–498.

31.   WORTHINGTON R.W., STEVENSON B.J. & DE LISLE G.W. (1985). Serology and semen culture for the diagnosis of *Brucella ovis* infection in chronically infected rams. *N.Z. Vet. J.,* **33**, 84–86.

32.   WORTHINGTON R.W., WEDDELL W. & PENROSE M.E. (1984). A comparison of three serological tests for the diagnosis of *B. ovis* infection in rams. *N.Z. Vet. J.,* **32**, 58–60.

*

*   *

NB: There are OIE Reference Laboratories for Ovine epididymitis (*Brucella ovis*) (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).

CHAPTER 2.8.5.

PORCINE BRUCELLOSIS

SUMMARY

Brucellosis in pigs is caused by Brucella suis*, a bacterial infection that, after an initial bacteraemia, causes chronic inflammatory lesions in the reproductive organs of both sexes, with occasional localisation and lesions in other tissues. The species* Brucella suis *consists of five biovars, but the infection in pigs is caused by* B. suis *biovars 1, 2 or 3. The disease caused by biovars 1 and 3 is similar, while that caused by biovar 2 differs from 1 and 3 in its host range, its limited geographical distribution and its pathology. Biovar 2 is rarely pathogenic for humans, whereas biovars 1 and 3 are highly pathogenic causing severe disease. Porcine brucellosis is of widespread occurrence; generally, however, the prevalence is low, with the exception of South America and South-East Asia where the prevalence is higher. In some areas,* B. suis *infection has become established in wild or feral pigs – diagnostic methods recommended for wild and feral pigs are the same as for domestic pigs. Various biovars of* B. suis *cause infections in animals other than pigs, such as reindeer, caribou, hares and various murine species, and occasionally in cattle and dogs.* Brucella suis *infections in animals other than pigs are dealt with in an Appendix at the end of this chapter.*

Signs of disease in sows include infertility and abortion at any stage of gestation, and birth of dead or weak piglets. In boars, the most prominent sign is orchitis, and the secondary sex organs may be affected. Brucella suis *may be present in the semen, sometimes in the absence of clinical signs. Transmission during copulation is more common than is the case with brucellosis in ruminants. In both sexes, bones and especially joints and tendon sheaths may be affected, causing lameness and sometimes paralysis. Pigs are susceptible to artificial infection with* B. abortus *and* B. melitensis*, but reports of natural disease in pigs being caused by either of these organisms are rare. In humans, the infection is usually confined to those who are occupationally exposed to pigs, and to laboratory workers. The capability of* B. suis *to colonise the bovine udder with subsequent shedding in milk, has the potential to be a serious human health risk.*

Identification of the agent: Brucella suis *is readily isolated from live pigs by culture of birth products, and from carcasses by culture of lymph nodes and organs. Selective media are available for culture of contaminated samples. In nature,* B. suis *occurs invariably in the smooth phase – the appearance on solid medium is typical of smooth brucellae. Biovars of porcine origin agglutinate with monospecific A antiserum, and not with M antiserum. Definite identification of species and biovars may be effected by phage typing, molecular and biochemical tests, preferably carried out in specialised laboratories.*

Serological tests: *To date, none of the serological tests has been shown to be reliable in routine diagnosis in individual pigs. The indirect and competitive enzyme-linked immunosorbent assays (*ELISAs*), as well as the Rose Bengal test (*RBT*), complement fixation test (*CFT*) and fluorescence polarisation assay (*FPA*) are the prescribed tests for international trade purposes. The allergic skin test and the buffered plate agglutination test (*BPAT*) are also useful for identifying infected herds. The procedures for all the tests are the same as those described in Chapter 2.4.3 Bovine brucellosis.*

Requirements for vaccines and diagnostic biologicals: Brucella suis *strain 2 vaccine has been used for immunising pigs in China (*People's Rep. of*). Confirmation of the results obtained in China is required before strain 2 vaccine can be recommended for general use. In other countries, experimental work has shown that* B. melitensis *Rev.1 vaccine is superior to* B. suis *strain 2 in protecting sheep against* B. melitensis*. Sufficient data is not available to conclude if* B. abortus *strain RB51 vaccine is efficacious in protecting swine against exposure to* B. suis*. In practice, no*

> *product has yet found general acceptance. Preparation, testing and use of an established allergen, brucellin is described.*

A. INTRODUCTION

Porcine brucellosis is an infection caused by biovar 1, 2 or 3 of *Brucella suis*. It occurs in many countries where pigs are raised. Generally, the prevalence is low, but in some areas, such as South America and South-East Asia, the prevalence is much higher. Porcine brucellosis may be a serious, but presently unrecognised, problem in some countries. *Brucella suis* biovar 1 infections have been reported from feral pigs in some of the southern States of the United States of America (USA), and in Queensland, Australia. In both countries, a number of human infections have been reported from people who hunt and handle material taken from feral pigs (21, 22).

The disease is generally transmitted by consumption of feed contaminated by birth and/or abortion products and uterine discharges. Pigs will readily eat aborted fetuses and placental membranes. Transmission during copulation also occurs frequently, and this has implications for those practising artificial insemination.

In pigs, as in ruminants, after the initial bacteraemia, *B. suis* colonises cells of the reproductive tract of either sex. In females, placentas and fetuses are invaded, while in males, invasion occurs in one or more of the following: testis, prostate, epididymis, seminal vesicles, and/or bulbo-urethral glands. In males the lesions, which are most often unilateral, start with a hyperplasia that may progress to abscess formation; the final stage is characterised by sclerosis and atrophy. Arthritis may occur in various joints, and sometimes spondylitis occurs.

The most common manifestation of brucellosis in female pigs is abortion, occurring very early or at any time during gestation. Vaginal discharge is not often evident, and, in chronically infected herds, infertility rather than abortion is the most relevant clinical sign of the disease. In males, brucellosis is more likely to be persistent, with lesions in the genital tract often leading to interference with sexual activity, which can be temporary or permanent. The boar may excrete brucellae in the semen without any apparent abnormality in the sex organs or interference with sexual activity.

In both sexes, there may be swollen joints and tendon sheaths, lameness and, occasionally, posterior paralysis. A significant proportion of both male and female pigs will recover from the infection, often within 6 months, but many will remain permanently infected.

Brucellosis caused by *B. suis* biovar 2 differs from infection caused by biovars 1 and 3 in its host range, its distribution, and in its pathology. In general, the geographical distribution of biovar 2 has historically been in a broad range between Scandinavia and the Balkans (2). The prevalence in wild boars appears to be high throughout continental Europe (1, 3, 12, 14, 15). In recent outbreaks in Europe, wild boars have been implicated as the source of transmission of biovar 2 to outdoor reared pigs (12). In addition to wild boars, the European hare (*Lepus capensis*) is also considered as a reservoir for *B. suis* biovar 2 and has been implicated as a possible source of transmission to domestic livestock (2, 14, 24). *Brucella suis* biovar 2 causes miliary lesions, particularly reproductive tissues, that often become purulent. To date, biovar 2 has rarely been reported as the cause of human brucellosis. However, biovar 2 infection has been reported in two immuno-compromised hunters, who had been extensively exposed through gutting or skinning boars or hares (13).

The most common *B. suis* biovars (1 and 3) are serious human pathogens and adequate precautions are needed when handling and disposing of potentially infective material. This is especially so in the laboratory after culture has greatly increased the number of organisms present. Laboratory manipulation of the cultures or contaminated material from infected animals must be done under strict biosafety conditions to safely handle this dangerous zoonotic agent. Biosafety containment level 3 is recommended (see Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities).

The classification, microbiological and serological properties of the genus *Brucella* and related species and biovars are given in the Chapter 2.4.3 Bovine brucellosis.

B. DIAGNOSTIC TECHNIQUES

As far as biovars 1 and 3 are concerned, culture methods are at least as sensitive as serology (6). Biovar 2 appears to be highly sensitive to selective media and could be more difficult to isolate (Garin-Bastuji & Blasco, unpublished data). As the product of almost all pig-raising enterprises passes through abattoirs, surveillance methods (serology and culture) can be applied effectively at this point. In many areas, traditional village pig breeding is now accompanied by the development of larger commercial units, thereby increasing the use of

artificial insemination. Whereas artificial insemination using brucellosis-free boars can be a valuable aid in the control of porcine brucellosis, the inadvertent use of infected semen could, obviously, cause incalculable damage.

1. Identification of the agent

Optimal samples for bacteriologic culture and methods for processing of samples are similar to those described for bovine brucellosis in Chapter 2.4.3 Bovine brucellosis. Standard and selective media used for other species of brucellae are suitable for *B. suis* (see Chapter 2.4.3 Bovine brucellosis). The addition of serum is not essential, but basal medium containing 5% serum is a satisfactory medium, both for isolation, maintenance of cultures and typing. The addition of CO_2 to the atmosphere is not required.

In nature, *B. suis* invariably occurs in the smooth form and colonies are morphologically indistinguishable from other smooth brucellae, described in Chapter 2.4.3 Bovine brucellosis.

Biovars 1, 2 and 3 of *B. suis* are all A surface antigen dominant, and growth may be presumptively identified by slide agglutination with the monospecific A antiserum. Confirmatory identification of species and biovar should be performed in a specialised reference laboratory. The OIE Reference Laboratories for brucellosis are listed in the Table given in Part 3 of this *Terrestrial Manual*.

Confirmation of species and biovar depends on phage tests, production of H_2S (only biovar 1 produces H_2S), and growth in the presence of dyes. However, some strains of *B. suis* biovar 1 are atypical in that they grow on media containing 20 µg/ml of basic fuchsin. Most strains of *B. suis* are inhibited by safranin O at a concentration of 1/10,000, whereas *B. suis* usually reacts more rapidly in the urease test than either *B. abortus* or *B. melitensis*. Oxidative metabolic tests are supplemental tests that can be used for distinguishing *B. suis* from other smooth *Brucella* species.

Molecular genetic techniques using the polymerase chain reaction (PCR) and specific primers are available that can identify *B. suis* and other species of *Brucella* (see chapter 2.4.3 Bovine brucellosis). Some of these techniques can distinguish biovars of *B. suis* (5, 7).

2. Serological tests

None of the serological tests used for the diagnosis of porcine brucellosis are reliable for diagnosis in individual pigs.

The major antigen involved in the serological tests currently available is the smooth lipopolysaccharide (LPS). The OPS moiety of this molecule contains epitopes that cross-react with those existing in the corresponding LPS from *Yersinia enterocolitica* serotype O:9 (16). Therefore, available serological tests are unable to distinguish between antibodies raised to these two infections. *Y. enterocolitica* O:9 infection in pigs is not uncommon in some areas (1, 26). Studies have suggested that the sensitivities and specificities of the Rose Bengal test (RBT), the indirect and competitive enzyme-linked immunosorbent assay (I- and C-ELISAs), and the fluorescent polarisation assay (FPA) are similar (20). With the exception of acute stages of infection (<9 weeks) (16), the use of the FPA (18) or C-ELISA has been reported to eliminate cross-reactivity with *Y. enterocolitica* but this should be confirmed in additional field studies performed in various epidemiological situations. Swine serum may sometimes also contain nonspecific antibody, thought to be of the IgM isotype, further reducing the specificity of conventional tests, especially the serum agglutination test (SAT). Also, swine complement interacts with guinea-pig complement to produce a pro-complementary activity that reduces the sensitivity of the complement fixation test (CFT). Sensitivity levels may be low for the CFT; therefore caution should be taken when interpreting test results from individual animals. For international and other trade, e.g. purchasing boars, the disease status of the herd and of the area in which the herd is situated are of more importance than tests on individual animals.

- **Reference sera**

  The OIE reference standards are those against which all other standards are compared and calibrated. For the RBT and CFT, please refer to Chapter 2.4.3 Bovine brucellosis for antigen standardisation and test protocols. A porcine reference standard for ELISAs and FPA has been developed and will be available to national laboratories in the near future[1].

a) **Enzyme-linked immunosorbent assay (prescribed tests for international trade)**

Indirect and competitive ELISAs have been developed for the diagnosis of brucellosis in individual pigs and for screening large numbers of sera. These techniques promise to be more efficient than any of the tests mentioned above. At this time the indirect ELISA and the reference sera for this test are not commercially available.

---

[1] Obtainable from the OIE Reference Laboratory for Brucellosis at the Ontario Laboratories (Fallowfield), Canadian Food Inspection Agencies, 3851 Fallowfield Road, Nepean, Ontario K2H 8P9, Canada.

- **Indirect ELISA**

A method for the I-ELISA is described in detail in Chapter 2.4.3 Bovine brucellosis, however, antibody specific for porcine IgG conjugated with horseradish peroxidase (HRPO) has to be used. The I-ELISA may be used as a screening test. Before being used for international trade, the cut-off of I-ELISA should be properly established using the appropriate validation techniques (see Chapter 1.1.4 Principles of validation of diagnostic assays for infectious diseases) and the test should be standardised against the above-mentioned Standard.

- **Competitive ELISA**

C-ELISA procedures for detection of porcine antibody to *Brucella* sp. (18) are identical to the procedures used for bovine antibody to *B. abortus* described in Chapter 2.4.3. This assay is capable of eliminating some reactions due to *Y. enterocolitica* O:9 and in some situations other cross-reacting antibody, such as IgM, will not compete well. Before being used for international trade, the cut-off of C-ELISA should be properly established using the appropriate validation techniques (see Chapter 1.1.4 Principles of validation of diagnostic assays for infectious diseases) and the test should be standardised against the above-mentioned Standard

b) Fluorescence polarisation assay (a prescribed test for international trade)

The FPA for detection of porcine antibody to *Brucella* sp. is essentially the same as that described for cattle (for more details see Chapter 2.4.3); an example serum dilution used is 1/25 for the tube test and 1/10 for the plate test (18). It is a simple technique for measuring antigen/antibody interaction and may be performed in the laboratory or in the field. Lyophilised porcine sera tend to increase background activity in this assay. The FPA may be used as a screening and/or confirmatory test. Before being used for international trade, the cut-off of FPA should have been properly established using the appropriate validation techniques (see Chapter 1.1.4 Principles of validation of diagnostic assays for infectious diseases) and the test should be standardised against the above-mentioned Standard.

c) Rose Bengal test (a prescribed test for international trade)

The preparation and standardisation of RBT antigen and the method of performing the test are described in Chapter 2.4.3 Bovine brucellosis. The *B. abortus* antigens are appropriate for testing swine sera in RBT and will identify antibody against all three biovars of *B. suis*. The RBT may be used as a screening test, bearing in mind its lack of specificity for discriminating reactions caused by brucellosis from those caused by infections by *Y. enterocolitica* O:9 and other cross-reacting bacteria.

d) Complement fixation test (a prescribed test for international trade)

The preparation and standardisation of CFT antigen and the method of performing the test are described in Chapter 2.4.3 Bovine brucellosis. The *B. abortus* antigens are appropriate for testing swine sera in CFT and will identify antibody against all three biovars of *B. suis*. The CFT has a reduced sensitivity for diagnosing *B. suis* infection and is not capable of eliminating all reactions caused by *Y. enterocolitica* O:9. It may be used as a complementary test.

3. Other Tests

a) Allergic (hypersensitivity) tests

Brucelin-INRA is an sLPS free cytosolic extract from rough *B. melitensis* B115. This preparation does not stimulate the formation of antibodies that would be reactive in BBAT, CFT or ELISAs. The product has been developed for use in ruminants, but is also effective for confirming the disease at the herd level in pigs. A rough strain is used in its preparation, thereby avoiding the presence of sLPS. The preparation, standardisation and testing, of Brucellin-INRA is described in detail in Chapter 2.4.3 Bovine brucellosis. As a diagnostic agent in pigs 0.1 ml of the allergen is injected intradermally into the skin at the base of the ear or preferably next to the base of the tail. The latter appears more practical and less hazardous. The reaction is read after 48 hours. A positive reaction shows erythema of non-pigmented skin and an oedematous swelling. In severe reactions, there may also be some necrosis.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Numerous attempts have been made to develop a vaccine to immunise pigs against *B. suis*, but none has been found fully effective. Only one immunogen -*B. suis* strain 2 (S2) vaccine- has been reported suitable after

extensive field use in south China (People's Rep. of) (17, 27)[2] but experiments under strictly controlled conditions are not available. To date, it does not appear to have been used elsewhere in pigs, probably because it has been shown that this vaccine does not confer adequate protection in sheep against *B. melitensis* (25). Similarly, the *B. abortus* strain RB51 vaccine has been reported as inefficacious in protecting swine against exposure to *B. suis* (23).

APPENDIX: *BRUCELLA SUIS* INFECTIONS IN ANIMALS OTHER THAN PIGS

1.   Rangiferine brucellosis

Brucella suis biovar 4 causes serious disease in reindeer or caribou (*Rangifer tarandus* and its various subspecies) throughout the Arctic region, including Siberia, Canada and Alaska (16, 19). Some of these animals are domesticated, others are wild and migratory. *Rangifer tarandus* is very susceptible to *B. suis* infection, which causes fever, depression and various local signs, such as abortion, retained placentas, metritis, sometimes with blood-stained discharge, mastitis, bursitis and orchitits. In the Arctic region, *B. suis* biovar 4 constitutes a serious zoonosis (8). Transmission to humans may be by direct contact or through consumption of milk and other inadequately heated products from reindeer. Bone marrow, which is considered to be a special delicacy in this region, is also a source of human infection.

The methods already described for isolating and identifying *B. suis* in samples taken from pigs are equally applicable to *B. suis* biovar 4 in samples taken from reindeer. Biovar 4 grows well on all the usual media used for the culture of *Brucella*. It reacts positively with both anti-A and -M monospecific sera. For serology, the tube agglutination test has been reported to be satisfactory, with titres of 1/20 or greater being considered to be diagnostic. The CFT has also been used but the clinical interpretation of these tests in reindeer has not been established.

Vaccination of reindeer with *B. abortus* S19 vaccine, or alternatively with *B. abortus* 45/20 adjuvant vaccine, has been tried experimentally without any clear-cut result. In the case of S19, the reaction to vaccination was rather severe and immunity in the vaccinated animals could only be demonstrated against challenge with very small doses of *B. suis* biovar 4. Gall *et al.* (9) compared several serological tests and found that the specificity values for the BPAT and CFT using reindeer/caribou sera was lower than the I-ELISA, C-ELISA and the FPA, while sensitivity values were similar for all tests.

2.   *Brucella suis* infection in other nonporcine species

There are two different types of epidemiological situation with regard to *B. suis* infection in other nonporcine species. In the first case, *B. suis* infection occurs in animals that are not the natural host of the particular infection through the ingestion of contaminated materials or by co-habitation with infected natural hosts. For example, Arctic foxes and wolves may contract *B. suis* biovar 4 from reindeer; dogs and rodents, such as rats and mice, may acquire other *B. suis* biovars by cohabitation with infected hosts. Cattle and horses may become infected by cohabitation or interaction with infected swine (4). The infecting bacteria are invariably the well defined biovars of the natural host species.

In the second case, wildlife species that are natural hosts for *B. suis* or *B.-suis*-like infections become infected. One example is the so-called murine brucellosis of the former USSR, where small rodents are infected with *B. suis* biovar 5. Other similar situations have been reported from Queensland, Australia and from Kenya. In all three cases, *B. suis* strains with different characteristics were involved, and at least one of them was difficult to classify.

Brucellosis caused by *B. suis* biovar 2 in the European hare (*Lepus capensis*) (24), is characterised by the formation of nodules, varying in size from that of a millet seed to a cherry or even larger; these often become purulent. Such nodules may occur in almost any location, sometimes subcutaneously or intramuscularly, in the spleen, liver or lung and in the reproductive organs of either sex. The bodily condition of the hare may be surprisingly unaffected. Other species may also become infected by cohabitation with *B. suis* biovar 2 infected swine, wild boars or hares. Gutting or skinning wild boars in cattle sheds could be a route of transmission to cattle (11).

Serological investigations in nonporcine species are usually carried out for screening purposes. In these particular circumstances, specificity is more important than sensitivity. Here the CFT can be recommended, although the RBT, may be also useful because of its simplicity. The indirect and competitive ELISAs appear to be very useful for epidemiological sero-surveys in wild boars as both are more sensitive and specific than the RBT and CFT. The

---

2   Obtainable from the National Institute for the Control of Veterinary Products and Pharmaceuticals, Ministry of Agriculture, 30 Baishiqiao Road, Beijing 100081, China (People's Rep. of), or from VLA Weybridge, New Haw, Addlestone, Surrey KT15 3NB, United Kingdom. Supply from the Weybridge laboratory needs the prior permission of the World Health Organization.

test has also been used successfully on blood samples that are in poor condition. When poor quality samples are tested on other tests, the results may be uninterpretable. Another advantage of the ELISAs is that if serum is not available, it is possible to test meat juice samples (10). However, in nonporcine species the interpretation of serological results may be problematic. Where positive or equivocal serological results are encountered, a bacteriological investigation should be conducted.

For bacteriological investigations in situations such as these, where the infecting organisms may have unusual characteristics, it is advisable to duplicate the culture on selective media by culture on plain medium supplemented with 5% serum, and to broaden the investigation by incubating the cultures in an atmosphere containing 10% CO_2. Colonies resembling *Brucella* can be tentatively identified by Gram staining, by slide agglutination tests with monospecific anti-A and -M sera, and by anti-rough *Brucella* serum (Chapter 2.4.3 Bovine brucellosis) *Brucella suis* biovar 5 is unusual in that it reacts with anti-M monospecific serum, and not with anti-A monospecific serum. Further identification is best carried out in a specialised laboratory.

REFERENCES

1. AL DAHOUK S., NOCKLER K., TOMASO H., SPLETTSTOESSER W.D., JUNGERSEN G., RIBER U., PETRY T., HOFFMANN D., SCHOLZ H.C., HENSEL A. & NEUBAUER H. (2005). Seroprevalence of brucellosis, tularaemia, and yersiniosis in wild boars (*Sus scrofa*) from north-eastern Germany. *J. Vet. Med.* [*B*]. *Infect. Dis. Vet. Public Health*, **22**, 444–455.

2. ALTON G.G. (1990). *Brucella suis. In:* Animal Brucellosis. Nielsen K. & Duncan J.R., eds. CRC Press, Boston, USA.

3. CVETNIC Z., MITAK M., OCEPEK M., LOJKIC M., TERZIC S., JEMERSIC L., HUMSKI A.,HABRUN B., SOSTARIC B., BRSTILO N., KRT B. & GARIN-BASTUJI B. (2003). Wild boars (*Sus scrofa*) as reservoirs of *Brucella suis* biovar 2 in Croatia. *Acta Vet. Hung.,* **51**, 465–473.

4. CVETNIC Z., SPICIC S., CURIC S., JUKIC B., LOJKIC M., ALBERT D., THIÉBAUD M. & GARIN-BASTUJI B. (2005). Isolation of *Brucella suis* biovar 3 from horses in Croatia. *Vet. Rec.*, **156**, 584–585.

5. FERRÃO-BECK L., CARDOSO R., MUÑOZ P.M., DE MIGUEL M.J., ALBERT D., FERREIRA A.C., MARÍN C.M., THIÉBAUD M., JACQUES I., GRAYON M., ZYGMUNT M.S., GARIN-BASTUJI B., BLASCO J.M. & SÁ M.I. (2006). Development of a multiplex PCR assay for polymorphism analysis of *Brucella suis* biovars causing brucellosis in swine, *Vet. Microbiol.,* **115**, 269–277.

6. FERRIS R.A., SCHOENBAUM M.A. & CRAWFORD R.P. (1995). Comparison of serologic tests and bacteriologic culture for detection of brucellosis in swine from naturally infected herds. *J. Am. Vet. Med. Assoc.*, **207**, 1332–1333.

7. FRETIN D., WHATMORE A.M., AL DAHOUK S., NEUBAUER H., GARIN-BASTUJI B., ALBERT D., VAN HESSCHE M., MÉNART M., GODFROID J., WALRAVENS K. & WATTIAU P. (2008). Rapid differentiation of *Brucella suis* biovars by real-time PCR. *Vet. Microbiol.* **131**, 376–385.

8. FORBES L.B. (1991). Isolates of *B. suis* biotype 4 from animals and humans in Canada, 1982–1990. *Can. Vet. J.*, **32**, 686–688.

9. GALL D., NIELSEN K., FORBES L., COOK W., LECLAIR D., BALSEVICIUS S., KELLY L., SMITH P. & MALLORY M. (2001). Evaluation of the fluorescence polarization assay and comparison to other serological assays for detection of brucellosis in cervids. *J. Wildl. Dis.*, **37**, 110–118.

10. GARIN-BASTUJI B., CAU C., HARS J., BOUE F. & TERRIER M.E. (2004). Utilisation comparée du sérum, du poumon et du muscle pour le dépistage de la brucellose chez les sangliers. *Epidémiol. Santé Anim.*, **45**, 13–23.

11. GARIN-BASTUJI B. & DELCUEILLERIE F. (2001). Les brucelloses humaine et animales en France en l'an 2000. Situation épidémiologique – Programmes de contrôle et d'éradication. *Méd. Mal. Infect.*, **31** Suppl 2, 202–216.

12. GARIN-BASTUJI B., HARS J., CALVEZ D., THIEBAUD M. & ARTOIS M. (2000). Brucellose du porc domestique et du sanglier sauvage due à *Brucella suis* biovar 2 en France. *Epidémiologie & Santé Animale*, **38**, 1–5.

13. GARIN-BASTUJI B., VAILLANT V., ALBERT D., TOURRAND B., DANJEAN M.P., LAGIER A., RISPAL P., BENQUET B., MAURIN M., DE VALK H. & MAILLES A. (2006). Is brucellosis due the biovar 2 of *Brucella suis* an emerging

zoonosis in France? Two case reports in wild boar and hare hunters. *In:* Proceedings of the International Society of Chemotherapy Disease Management Meeting, 1st International Meeting on Treatment of Human Brucellosis, 7–10 November 2006, Ioannina, Greece.

14. GODFROID J. & KASBOHRER A. (2002). Brucellosis in the European Union and Norway at the turn of the twenty-first century. *Vet. Microbiol.*, **90**, 135–145.

15. GODFROID J., MICHEL P., UYTTERHAIEGEN L., DE SMEDT C., RASSENEUR F., BOELAERT F., SAEGERMAN C. & PATIGNY X. (1994). Brucellose enzootique a *Brucella suis* biotype 2 chez le sanglier *(Sus scrofa)* en Belgique. *Ann. Med. Vet.*, **138**, 263–268.

16. JUNGERSEN G., SØRENSEN V., GIESE S.B., STACK J.A. & RIBER U. (2006). Differentiation between serological responses to *Brucella suis* and *Yersinia enterocolitica* O:9 after natural or experimental infection in pigs. *Epidemiol. Infect.*, **134**, 347–357.

17. LIANG XINGXIAN (1991). The prevalence, prevention and control of swine brucellosis in Guangdong Province, China. Document AGU/BRU/92/21 Food and Agriculture Organization of the United Nations (FAO), Rome, Italy.

18. NIELSEN K., GALL D., SMITH P., VIGLIOCCO A., PERREZ B., SAMARTINO L., DAJER A., ELZER P. & ENRIGHT F. (1999). Validation of the fluorescence polarization assay as a serological test for the presumptive diagnosis of porcine brucellosis. *Vet. Microbiol.*, **68**, 245–253.

19. ORLOW E.S. (1963). Brucellosis in reindeer. Proceedings of the 17th World Veterinary Congress, Hanover, Germany, 1, 585–588.

20. PAULO P.S., VIGLIOCCO A.M., RAMONDINO R.F., MARTICORENA D., BISSI E., BRIONES G., GORCHS C., GALL D. & NIELSEN K. (2000). Evaluation of primary binding assays for presumptive serodiagnosis of swine brucellosis in Argentina. *Clin. Diagn. Lab. Immunol.*, **7**, 828–831.

21. ROBSON J.M., HARRISON M.W., WOOD R.N., TILSE M.H., MCKAY A.B. & BRODRIBB T.R. (1993). Brucellosis: reemergence and changing epidemiology in Queensland. *Med. J. Aust.*, **159**, 153–158.

22. STARNES C.T., TALWANI R., HORVATH J.A., DUFFAS W.A., & BRYAN C.S. (2004). Brucellosis in two hunt club members in South Carolina. *JSC Med. Assoc.*, **100**, 113–115.

23. STOFFREGEN W.C., OLSEN S.C., BRICKER, B.J. (2006). Parenteral vaccination of domestic pigs with *Brucella abortus* strain RB51. *Am. J. Vet. Res.*, **67**, 1802-1808.

24. SZULOWSKI K., IWANIAK W., PILASZEK J., TRUSZCZYNSKI M. & CHROBOCINSKA M. (1999). The ELISA for the examination of hare sera for anti-Brucella antibodies. *Comp. Immunol. Microbiol. Infect. Dis.*, **22,** 33–40.

25. VERGER J.M., GRAYON M., ZUNDEL E., LECHOPIER P. & OLIVER-BERNARDIN V. (1995). Comparison of the efficacy of *Brucella suis* strain 2 and *Brucella melitensis* Rev.1 live vaccines against a *Brucella melitensis* experimental infection in pregnant ewes. *Vaccine*, **13**, 191–196.

26. WRATHALL A.E., BROUGHTON E.S., GILL K.P.W. & GOLDSMITH G.P. (1983). Serological reactions to *Brucella* species in British pigs. *Vet. Rec.*, **132**, 449–454.

27. XIE XIN (1986). Orally administrable brucellosis vaccine: *Brucella suis* strain 2 vaccine. *Vaccine*, **4**, 212–216.

*

* *

NB: There are OIE Reference Laboratories for Porcine brucellosis (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).