

## CHAPTER 2.4.3.

# BOVINE BRUCELLOSIS

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### 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 guanicoe*), and vicuna (*Vicugna vicugna*) 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 <sup>b</sup>	Serum requirement	Lysis by phages <sup>a</sup>					Oxidase	Urease activity	Preferred host
			Tb		Wb	Iz <sub>1</sub>	R/C			
			RTD <sup>c</sup>	10 <sup>4</sup> RTD	RTD	RTD	RTD			
<i>B. abortus</i>	S	– <sup>d</sup>	+	+	+	+	–	+ <sup>e</sup>	+ <sup>f</sup>	Cattle and other Bovidae
										Biovar 1: swine
										Biovar 2: swine, hare
<i>B. suis</i>	S	–	–	+	+ <sup>g</sup>	+ <sup>g</sup>	–	+	+ <sup>h</sup>	Biovar 3: swine
										Biovar 4: reindeer
										Biovar 5: wild rodents
<i>B. melitensis</i>	S	–	–	–	– <sup>i</sup>	+	–	+	+ <sup>j</sup>	Sheep and goats
<i>B. neotomae</i>	S	–	– <sup>k</sup>	+	+	+	–	–	+ <sup>h</sup>	Desert wood rat <sup>l</sup>
<i>B. ovis</i>	R	+	–	–	–	–	+	–	–	Rams
<i>B. canis</i>	R	–	–	–	–	–	+	+	+ <sup>h</sup>	Dogs
<i>B. ceti</i>	S		+ <sup>m</sup>		+ <sup>n</sup>	+ <sup>o</sup>	–	+	+	Cetaceans
<i>B. pinnipedialis</i>	S		+ <sup>m</sup>		+ <sup>n</sup>	+ <sup>o</sup>	–	+	+	Pinnipeds
<i>B. microti</i>	S	–	–	+	+	+	–	+	+	Common vole

From refs 1, 39.

- a Phages: Tbilisi (Tb), Weybridge (Wb), Izatnagar1(Iz<sub>1</sub>) 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<sub>1</sub>
- 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 <sub>2</sub> requirement	H <sub>2</sub> S production	Growth on dyes <sup>a</sup>		Agglutination with monospecific sera		
				Thionin	Basic fuchsin	A	M	R
<i>B. melitensis</i>	1	–	–	+	+	–	+	–
	2	–	–	+	+	+	–	–
	3	–	–	+	+	+	+	–
<i>B. abortus</i>	1	+ <sup>b</sup>	+	–	+	+	–	–
	2	+ <sup>b</sup>	+	–	–	+	–	–
	3	+ <sup>b</sup>	+	+	+	+	–	–
	4	+ <sup>b</sup>	+	–	+ <sup>c</sup>	–	+	–
	5	–	–	+	+	–	+	–
	6	–	–	+	+	+	–	–
	9	+ or –	+	+	+	–	+	–
<i>B. suis</i>	1	–	+	+	– <sup>d</sup>	+	–	–
	2	–	–	+	–	+	–	–
	3	–	–	+	+	+	–	–
	4	–	–	+	– <sup>e</sup>	+	+	–
	5	–	–		–	–	+	–
<i>B. neotomae</i>	–	–	+	– <sup>f</sup>	–	+	–	–
<i>B. ovis</i>	–	+	–	+	– <sup>e</sup>	–	–	+
<i>B. canis</i>	–	–	–	+	– <sup>e</sup>	–	–	+
<i>B. ceti</i>	–	–	–	+	+	+	– <sup>e</sup>	–
<i>B. pinnipedialis</i>	–	+	–	+	+	+	– <sup>e</sup>	–
<i>B. microti</i>	–	–	–	+	+	–	+	–

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<sub>2</sub> (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<sub>2</sub> 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<sub>2</sub> requirement for growth, production of H<sub>2</sub>S (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 <sup>2+</sup> (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 <sub>2</sub> 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

3 minutes of primer extension at 72°C

for a total of 25 cycles

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 <sup>a</sup>	Sequence (5'–3')	Amplicon size (bp)	DNA targets	Source of genetic differences
BMEI0998f  BMEI0997r	ATC-CTA-TTG-CCC-CGA-TAA-GG  GCT-TCG-CAT-TTT-CAC-TGT-AGC	1682	Glycosyltransferase, gene <i>wboA</i>	IS711 insertion in BMEI0998 in <i>B. abortus</i> RB51, and deletion of 15,079 bp in BMEI0993-BMEI1012 in <i>B. ovis</i>
BMEI0535f  BMEI0536r	GCG-CAT-TCT-TCG-GTT-ATG-AA  CGC-AGG-CGA-AAA-CAG-CTA-TAA	450 (1320 <sup>b</sup> )	Immunodominant antigen, gene <i>bp26</i>	IS711 insertion in BMEI0535-BMEI0536 in <i>Brucella</i> strains isolated from marine mammals
BMEI0843f  BMEI0844r	TTT-ACA-CAG-GCA-ATC-CAG-CA  GCG-TCC-AGT-TGT-TGT-TGA-TG	1071	Outer membrane protein, gene <i>omp31</i>	deletion of 25,061 bp in BMEI0826-BMEI0850 in <i>B. abortus</i>
BMEI1436f BMEI1435r	ACG-CAG-ACG-ACC-TTC-GGT-AT TTT-ATC-CAT-CGC-CCT-GTC-AC	794	Polysaccharide deacetylase	deletion of 976 bp in BMEI1435 in <i>B. canis</i>
BMEI0428f  BMEI0428r	GCC-GCT-ATT-ATG-TGG-ACT-GG  AAT-GAC-TTC-ACG-GTC-GTT-CG	587	Erythritol catabolism, gene <i>eryC</i> (D-erythrulose-1-phosphate dehydrogenase)	deletion of 702 bp in BMEI0427-BMEI0428 in <i>B. abortus</i> S19
BR0953f  BR0953r	GGA-ACA-CTA-CGC-CAC-CTT-GT  GAT-GGA-GCA-AAC-GCT-GAA-G	272	ABC transporter binding protein	deletion of 2653 bp in BR0951-BR0955 in <i>B. melitensis</i> and <i>B. abortus</i>
BMEI0752f BMEI0752r	CAG-GCA-AAC-CCT-CAG-AAG-C GAT-GTG-GTA-ACG-CAC-ACC-AA	218	Ribosomal protein S12, gene <i>rpsL</i>	point mutation in BMEI0752 in <i>B. melitensis</i> Rev.1
BMEI0987f  BMEI0987r	CGC-AGA-CAG-TGA-CCA-TCA-AA  GTA-TTC-AGC-CCC-CGT-TAC-CT	152	Transcriptional regulator, CRP family	deletion of 2,203 bp in BMEI0986-BMEI0988 in <i>B. neotomae</i>

<sup>a</sup>Designations are based on the *B. melitensis* (BME) or *B. suis* (BR) genome sequences. f: forward; r: reverse.

<sup>b</sup>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 *omp25*, 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<sub>2</sub> 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<sup>1</sup>. The use of these promotes international harmonisation of diagnostic testing and antigen standardisation (97):

<sup>1</sup> 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<sub>SP</sub>SS), a weak positive (OIEELISA<sub>WP</sub>SS) and a negative (OIEELISA<sub>N</sub>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)<sup>2</sup> 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<sub>2</sub>-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

<sup>2</sup> 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  $3.65 \pm 0.05$ . 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**

- Bring the serum samples and antigen to room temperature ( $22 \pm 4^\circ\text{C}$ ); only sufficient antigen for the day's tests should be removed from the refrigerator.
- Place 25–30  $\mu\text{l}$  of each serum sample on a white tile, enamel or plastic plate, or in a WHO haemagglutination plate.
- Shake the antigen bottle well, but gently, and place an equal volume of antigen near each serum spot.
- 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.
- 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).
- 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  $3.70 \pm 0.03$  and the pH of a serum:antigen mixture at a ratio of 8:3 should be  $4.02 \pm 0.04$ . 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**

- Bring the serum samples and antigen to room temperature ( $22 \pm 4^\circ\text{C}$ ); only sufficient antigen for the day's tests should be removed from the refrigerator.
- Shake the sample well. Place 80  $\mu\text{l}$  of each serum sample on a glass plate marked in  $4 \times 4$  cm squares
- Shake the antigen bottle well, but gently, and place 30  $\mu\text{l}$  of antigen near each serum spot.
- 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.
- 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
- The plate should be removed and rotated as above, and then returned for a second 4-minute incubation
- 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  $37^\circ\text{C}$  for 30 minutes or  $4^\circ\text{C}$  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  $37^\circ\text{C}$  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 ( $\text{C}'\text{H}$  or  $\text{MHD}_{50}$  or  $\text{C}'\text{H}$  or  $\text{MHD}_{100}$ ), respectively. It is generally recommended to titrate the complement before each set of tests, a macromethod being preferred for an optimal determination of  $\text{C}'\text{H}_{50}$ . Usually, 1.25–2  $\text{C}'\text{H}_{100}$  or 5–6  $\text{C}'\text{H}_{50}$  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:  $1000 \times 1/200 \times \text{titre of test serum} = \text{number of ICFTU of antibody in the test serum per ml}$ . 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<sub>1</sub> 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<sub>1</sub> 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 dialysed 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)**

- 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.
- 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.
- 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.
- 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<sub>1</sub> 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.
- Unbound conjugate is removed by four washing steps. Volumes (100 µl) of substrate/chromogen (1.0 mM H<sub>2</sub>O<sub>2</sub> [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.
- 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:

$$\text{Per cent positivity (\%P)} = \text{absorbance (test sample)} / \text{absorbance (strong positive control)} \times 100$$

The sLPS antigen, small amounts of the MAb specific for the heavy chain of bovine IgG<sub>1</sub>, software for generation of data using particular spectrophotometers and a standard test protocol for the I-ELISA are available for research and standardisation purposes<sup>3</sup>.

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**

- 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.
- 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.
- 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.
- 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.
- Unbound conjugate is removed by four washing steps. Volumes (100 µl) of substrate/chromogen (1.0 mM H<sub>2</sub>O<sub>2</sub> 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.
- 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:

$$\text{Per cent inhibition (\%I)} = 100 - (\text{absorbance [test sample]}/\text{absorbance [buffer control]} \times 100)$$

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**

- 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.
- A volume of antigen, which results in a total fluorescence intensity of 250–300 × 10<sup>3</sup>, 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.
- A reading above the predetermined threshold is indicative of a positive reaction.
- 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<sup>4</sup>.

- **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<sup>5</sup> 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 Montañana 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 ( $20 \pm 3^\circ\text{C}$ ); 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  $\mu\text{l}$  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 than 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  $\mu\text{l}$  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<sup>6</sup> 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-INRA 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

<sup>6</sup> 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<sup>7</sup> 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<sup>8</sup>. 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

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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 S19 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  $5-8 \times 10^{10}$  viable organisms. A reduced dose of from  $3 \times 10^8$  to  $3 \times 10^9$  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  $5 \times 10^9$  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  $1-3.4 \times 10^{10}$  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  $1-3 \times 10^9$  viable strain RB51 organisms (66, 83). In other countries, it is recommended to vaccinate cattle as calves (4–12 months of age) with a  $1-3.4 \times 10^{10}$  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 ( $1 \times 10^9$  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<sub>2</sub>-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<sup>9</sup>. 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.

<sup>9</sup> 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  $24 \times 10^9$  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  $35^\circ\text{C}$  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  $4^\circ\text{C}$ .

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  $50 \times 10^9$  per standard subcutaneous dose ( $5 \times 10^9$  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  $1\text{--}3.4 \times 10^{10}$  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  $5 \times 10^9$  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  $1 \times 10^8$  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<sub>2</sub> 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<sub>2</sub>PO<sub>4</sub> 1.0 g; K<sub>2</sub>HPO<sub>4</sub> 2.0 g; distilled water 1000 ml; pH 6.8) and adjust the suspension in BSS to 10<sup>9</sup> 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<sup>8</sup> 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<sub>50</sub>) by the SAS® statistical method specifically developed for RT<sub>50</sub> 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  $7.0 \pm 1.3$  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  $2 \times 10^5$  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  $-20^\circ C$  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  $37^\circ C$  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  $1 \times 10^4$  *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 *wboA* 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% sucrose 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 gentamycin 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.

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**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](http://www.oie.int)).