CHAPTER 3.8.7.

OVINE EPIDIDYMITIS
(*BRUCELLA OVIS*)

SUMMARY

Brucella ovis *infects sheep causing a clinical or subclinical disease that is characterised by genital lesions and reduced fertility in rams, placentitis and abortions in ewes, and increased perinatal mortality in lambs. The disease has been reported in American and European countries as well as Australia, New Zealand and South Africa, but probably occurs in most sheep-raising countries.*

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

Serological tests: The complement fixation test (CFT), agar gel immunodiffusion (AGID) test and indirect enzyme-linked immunosorbent assays (I-ELISA) using soluble surface antigens obtained from the B. ovis REO 198 strain, should be used for diagnosis. The sensitivities of the AGID test and I-ELISA are similar and may be higher than that the CFT. A combination in parallel of the AGID test and I-ELISA seems to give the best results in terms of sensitivity, but with regard to simplicity and cost, the AGID test is the most practicable test for diagnosing B. ovis *in non-specialised laboratories. However, because of the lack of standardised methods recognised at the international level for AGID and I-ELISA, the test that is most suitable for certifying individual animals prior to movement, including for international trade, remains the CFT.*

Requirements for vaccines: Seed cultures for vaccine production should be obtained from internationally recognised laboratories. A single standard dose (10^9 colony-forming units) of the live B. melitensis *Rev.1 vaccine, administered subcutaneously or, better, conjunctivally, can be used safely and effectively in rams, for the prevention of* B. ovis *infection. This vaccine strain should meet minimal quality standards: safe in the host animal, of adequate concentration, with absence of dissociation, adequate residual virulence and possess immunogenicity (efficacy) and be free of extraneous agents (see Chapter 3.1.4 Brucellosis [Brucella abortus, B. melitensis and B. suis]).*

A. INTRODUCTION

1. Definition of the disease

Brucella ovis produces a disease unique to sheep and is one of the most common causes of epididymitis in rams and a rare cause of infertility and abortion in ewes and neonatal mortality in lambs.

2. Causal pathogen

Brucella ovis and *B. canis* are the two presently known *Brucella* species naturally in the rough phase. *Brucella ovis* is similar to the other *Brucella* spp. in its morphology, staining properties and cultural characteristics, except that it gives negative reactions to the oxidase and urease tests. The microbiological and serological properties of all

Brucella species and biovars are given in detail in the Chapter 3.1.4 *Brucellosis* (Brucella abortus, B. melitensis *and* B. suis).

3.   Description of the disease

Brucella ovis infects sheep causing genital lesions (epididymitis and orchi-epididymitis) and infertility in rams, placentitis, abortions and infertility in ewes, and increased perinatal mortality in lambs. *Brucella ovis* is usually excreted in semen in infected rams. Passive venereal transmission via the ewe appears to be the most frequent route of infection, but ram-to-ram transmission is also very common (Blasco, 1990; 2010). Under the semi-extensive production systems (most common in European Mediterranean countries), rams are usually housed together. Direct ram-to-ram transmission during non-breeding periods is thus quite frequent and has been suggested to take place by several routes, including anal intercourse and, more frequently, through oral-genital contact (preputial licking).

Moreover, infected ewes may excrete *B. ovis* in vaginal discharges and milk and, accordingly, ewe-to-ram and lactating ewe-to-lamb transmission could also be determinant mechanisms of infection. Accordingly, ewes should be considered relevant in the epidemiology of infection, and this should be taken into account for the effective eradication of *B. ovis* in infected flocks (Blasco, 2010; Grilló *et al.,* 1999).

The disease has been reported in American and European countries as well as Australia, New Zealand and South Africa, but probably occurs in most sheep-raising countries.

The demonstration of genital lesions (unilateral or bilateral epididymitis and orchi-epididymitis) by palpating the testicles of rams may suggest the presence of this infection in a given flock. However, clinical diagnosis lacks sensitivity because not all rams infected with *B. ovis* present palpable genital lesions (Blasco, 1990). Moreover, clinical diagnosis lacks specificity since many other bacteria may cause genital lesions in rams. The most frequently reported pathogens such lesions in rams include *Actinobacillus seminis, A. actinomycetemcomitans, Histophilus ovis, Haemophilus* spp., *Corynebacterium pseudotuberculosis ovis, B. melitensis, Chlamydia abortus* and *Pasteurella* spp. (Bulgin & Anderson, 1983; Garcia-Pastor *et al.,* 2009; Livingstone & Hardy, 1964). Moreover, it must be emphasised that many palpable testicular lesions in rams are sterile, trauma-induced spermatic granulomas.

Although cattle, goats and deer have been proved susceptible to *B. ovis* in artificial transmission experiments, natural cases have been reported only in red deer reared in close contact with infected rams (Ridler *et al.,* 2012).

4.   Zoonotic risk and biosafety requirements

To date, no human cases have been reported, and *B. ovis* is considered to be non-zoonotic. However, in areas where *B. melitensis* infection co-exists with *B. ovis*, special care is required when handling samples, which should be transported to the laboratory in leak-proof containers (for further details see Chapter 3.1.4 Brucellosis (*Brucella abortus, B. melitensis* and *B. suis*) and Chapter 1.1.3 *Transport of biological materials*). All laboratory manipulations with live cultures or potentially infected/contaminated material must be performed at an appropriate biosafety and containment level determined by biorisk analysis (Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of infection with Brucella ovis

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement[a]	Contribute to eradication policies[b]	Confirmation of clinical cases[c]	Confirmation of suspect cases[d]	Herd/flock prevalence of infection – Surveillance
Identification of the agent[e]						
Staining methods	–	–	–	+	–	–
Culture	–	–	–	+++	+/++[d]	–
PCR[f]	–	–	–	+/++	+/++	–
Detection of immune response						
CFT	+++	+++	+++	++	++	++
I-ELISA	+++	+++	+++	++	++	+++
AGID	++	++	+++	++	++	++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; CFT = complement fixation test; I-ELISA = indirect enzyme-linked
immunosorbent assay; AGID = agar gel immunodiffusion test.
[a]This applies only to herds/flocks, countries or zones free from infection with *Brucella ovis*.
[b]To improve the efficiency of eradication policies in infected herds/flocks it is recommended to associate tests in parallel to increase the sensitivity of the diagnosis, i.e. two serological tests at least, e.g. CFT (or AGID) and I-ELISA;
[c]In low-prevalence or almost-free zones, the predictive value of positive results to serological tests may be very low. In such situation, the agent identification is usually needed for confirming clinical cases. In infected herds/flocks, a positive result to any serological test may be considered as a confirmation of a clinical case.
[d]In infected herds/flocks, any reactor in any serological test should be considered as infected. In low-prevalence or almost-free zones, singleton serological reactors may be confirmed by culture (and/or PCR). In free countries or zones, suspect animals are those positive to both a screening and a confirmatory serological test (tests in series, e.g. I-ELISA and CFT respectively) and should be confirmed by culture (and/or PCR).
[e]A combination of agent identification methods applied on the same clinical sample is recommended.
[f]False-positive results may occur.

1.   Identification of the agent

1.1.   Collection of samples

The most valuable samples for the isolation of *B. ovis* from live animals are semen, vaginal swabs and milk. For the collection of vaginal swabs and milk, see the instructions given in Chapter 3.1.4. Semen (genital fluids) can be collected in swabs taken from the preputial cavity of rams after electro-ejaculation. Alternatively, swabs can be taken directly from the vagina of brucellosis-free ewes immediately after being mated by the suspect ram. Clinically or sub-clinically infected rams may excrete *B. ovis* intermittently in their semen for years (Blasco, 2010). Vaginal swabs taken after abortion or premature lambing and milk samples are highly recommended samples to isolate *B. ovis* from infected ewes (Grilló *et al.,* 1999).

For the isolation of *B. ovis* after necropsy, the preferred organs in terms of probability of isolation are the epididymides, seminal vesicles, ampullae, and inguinal lymph nodes in rams, and the uterus, iliac and supra-mammary lymph nodes in ewes. However, to obtain maximum sensitivity, a complete search that

includes other organs and lymph nodes (spleen, cranial, scapular, pre-femoral and testicular lymph nodes) should be performed (Blasco, 2010). Dead lambs and placentas can also be examined. The preferred culture sites in aborted or stillborn lambs are abomasal content and lung.

Samples for culture should be refrigerated and transported to the laboratory to be cultured as soon as possible after collection. The organism remains viable for 48–72 hours at room temperature but if culture has to be delayed survival is enhanced by refrigerating or, preferably, freezing the tissue samples.

1.2. Staining methods

Semen or vaginal smears from clinically affected animals can be examined following staining by Stamp's method (Alton *et al.,* 1988) (see Chapter 3.1.4), and characteristic coccobacilli can be demonstrated in many infected animals. Examination of Stamp-stained smears of suspect tissues (ram genital tract, inguinal lymph nodes, placentas, and abomasal content and lung of fetuses) may also allow a rapid presumptive diagnosis. However, other bacteria with similar morphology or staining characteristics (*B. melitensis, Coxiella burnetii*, and *Chlamydia abortus*) can also be present in such samples, making the diagnosis difficult for inexperienced personnel. For such reason, microscopy results should always be confirmed by culture of the microorganism.

1.3. Culture

Due to its specificity, the isolation and identification of *B. ovis* in sheep fluids and tissues is the best direct method of diagnosis and, if positive, the only incontestable demonstration of *B. ovis* infection in a given animal or flock. Semen, vaginal swabs, or milk samples can be smeared directly onto plates containing adequate culture media and incubated at 37°C ± 2°C in an atmosphere of 5–10% CO_2. Tissues should be macerated and ground in a small amount of sterile saline or phosphate buffered saline (PBS) with a stomacher or blender, before plating. It is important to take into account that the larger the amount of tissue homogenates and the higher number of culture plates inoculated per diagnostic sample, the higher will be the final diagnostic sensitivity obtained.

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

Brucella ovis can be isolated in non-selective media, such as blood agar base enriched with 10% sterile ovine or bovine serum, or in blood agar medium with added 5–10% sterile ovine blood. However, since primary isolation requires 4–7 days of incubation, overgrowing fungi and commensal and environmental bacteria frequently contaminate the non-selective culture plates, and result in a reduced diagnostic sensitivity. Thus, the use of selective culture media is of paramount importance for a proper bacteriological diagnosis of *B. ovis* infection. The modified Farrell's selective medium used widely for the isolation of the smooth *Brucella* (see Chapter 3.1.4), inhibits the growth of *B. ovis* and should not be used (Marin *et al.,* 1996). Various selective media have been described, but modified Thayer–Martin's (mTM) medium (Marin *et al.,* 1996) has been used classically for isolating *B. ovis*. Briefly, this medium can be prepared with GC medium base (38 g/litre Difco, USA) supplemented with haemoglobin (10 g/litre) and colistin methane-sulphonate (7.5 mg/litre), vancomycin (3 mg/litre), nitrofurantoin (10 mg/litre), nystatin (100,000 International Units [IU]/litre = 17.7 mg) and amphotericin B (2.5 mg/litre). Working solutions are prepared as follows:

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

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

Antibiotic solutions (*prepared freshly*): colistin, nystatin and vancomycin are suspended in a mixture of methanol/water (1/1); nitrofurantoin is suspended in 1 ml of a 0.1 M NaOH sterile solution. For amphotericin B, it is recommended to prepare a stock solution of 10 mg/ml amphotericin B with 10 mg dissolved first in 1 ml sterile dimethyl sulphoxide (C_2H_6OS, analytical grade) and then added to 9 ml of sterile PBS (10 mM, pH 7.2 ± 0.1). Any stock solution remaining can be stored 5 days at 5°C ± 3°C. All

antibiotic solutions must be filtered through 0.22 μm filters before addition to the culture medium. Another suitable, but less effective, antibiotic combination can be: vancomycin (3 mg/litre); colistin (7.5 mg/litre); nystatin (12,500 IU/litre); and nitrofurantoin (10 mg/litre).

Once autoclaved, stabilise the temperature (45–50°C) of both solutions A and B with continuous stirring. Mix both solutions (adding A to B), avoiding bubble formation. Add the antibiotic solutions while stirring continuously and carefully, then distribute into sterile plates. Once prepared, the plates should not be stored for long periods, and freshly prepared medium is always recommended.

However, the mTM is not translucent due to the haemoglobin incorporated as a basal component, being thus unsuitable for the direct observation of colonial morphology. This has important practical consequences since this is probably the most widely used procedure for the presumptive identification of *Brucella* (Alton *et al.,* 1988). Having this in consideration, a new culture medium (named CITA) has been recently formulated using blood agar base as a basal component, and supplemented with 5% of sterile calf serum and the following antibiotics: vancomycin (20 mg/litre), colistin methanesulfonate (7.5 mg/litre), nitrofurantoin (10 mg/litre), nystatin (100,000 IU/litre), and amphotericin B (4 mg/litre). This antibiotic mixture can be prepared as indicated above for the preparation of the mTM medium. This new CITA medium inhibits most contaminant microorganisms but allows the growth of all *Brucella* species. Moreover, CITA medium outperforms mTM for isolation of *B. ovis*, and is more sensitive than both mTM and Farrell's media for isolating all smooth *Brucella* species from field samples, and is therefore the selective medium of choice for general *Brucella* isolation (De Miguel *et al.,* 2011).

All culture media used should be subjected to quality control with reference strains, to demonstrate that it performs properly.

1.4. Identification and typing

Brucella ovis colonies are not haemolytic. They are circular, convex, have unbroken edges, are always of the rough type when examined by oblique illumination, and test positive in the acriflavine test (Alton *et al.,* 1988). For growth, *B. ovis* needs an incubating atmosphere containing 5–10% CO_2. It lacks urease activity, fails to reduce nitrate to nitrite, and is oxidase negative. It does not produce H_2S and, although it does not grow in the presence of methyl violet, it usually grows in the presence of standard concentrations of thionin. The cultures are not lysed by *Brucella*-phages of the Tbilisi (Tb), Weybridge (Wb) and Izatnagar (Iz$_1$) groups at the routine test dilution (RTD) or 10^4 RTD, while they are lysed by phage R/C (Alton *et al.,* 1988). Most laboratories are not equipped enough for a complete identification of *Brucella* at species and biovar levels, and a practical schedule for presumptive identification is needed. Most *B. ovis* isolates can be correctly identified on the basis of growth characteristics, direct observation using obliquely reflected light, Gram or Stamp's staining, catalase, oxidase, urease and acriflavine tests. However, it is recommended that the definitive identification be carried out by reference laboratories with experience in identification and typing of *Brucella*.

The polymerase chain reaction (PCR) and other recently developed molecular methods provide additional means of detection and identification of *Brucella* sp. (see Chapter 3.1.4), and are becoming routine in many diagnostic laboratories. The existence of semen samples heavily contaminated with overgrowing organisms or containing dead *B. ovis*, could also justify the use of PCR as a supplementary direct diagnostic test. In fact, several PCR procedures have been reported to have similar sensitivity to standard bacteriological culture when applied to semen samples from *B. ovis* infected rams (Xavier *et al.,* 2010). However, the sensitivity and specificity of these PCR-based direct diagnostic procedures remain to be properly determined on other clinical samples and, for the moment, classical bacteriology should be considered the method of choice for the bacteriological diagnosis of *B. ovis*. By contrast, the use of the Bruce-ladder multiplex PCR (see Chapter 3.1.4) on DNA samples extracted from culture plate colonies is a rapid and highly specific procedure for the proper identification of most *Brucella* species including *B. ovis*.

2. Serological tests

The most efficient and widely used tests are the complement fixation test (CFT), the double agar gel immunodiffusion (AGID) test and the indirect enzyme-linked immunosorbent assay (I-ELISA). Several countries have adopted various standard diagnostic techniques for *B. ovis*, but the only test that is recognised by WOAH and the European Union (EU) as the test that is most suitable for certifying individual animals prior to movement,

including for international trade is the CFT. However, it has been demonstrated that the AGID test shows similar sensitivity to the CFT, and it is a simpler test to perform. Moreover, although international standardisation is lacking, numerous independent validation studies have shown that the I-ELISA is more sensitive than either the CFT or AGID test. AGID test and I-ELISA have been reported as more sensitive than the CFT. Conversely I-ELISA was sometimes reported as a less specific method, but this greatly depends on the protocol used (Estein *et al.*, 2002; Nielsen *et al.*, 2004; Praud *et al.*, 2012).

The International Standard anti-*Brucella ovis* Serum (ISaBoS, International Standard 1985[1]) is the one against which all other standards are compared and calibrated. This reference standard is available to national reference laboratories and should be used to establish secondary or national standards against which working standards can be prepared and used in the diagnostic laboratory for daily routine use.

HS antigens for use in serological tests should be prepared from. *Brucella ovis* strain REO 198[2] is CO_2- and serum-independent.

2.1. Antigens

When rough *Brucella* cells are heat-extracted with saline (hot-saline method, HS), they yield water-soluble antigenic extracts, the major component of which precipitates with sera to rough *Brucella* (Diaz & Bosseray, 1973; Myers *et al.*, 1972). For this reason, the HS extract has been referred to as the 'rough-specific antigen' or, when obtained from *B. ovis*, as the '*B. ovis*-specific antigen'. However, chemical characterisation of the HS extract from *B. ovis* has shown that it is enriched in rough lipopolysaccharide (R-LPS), group 3 outer membrane proteins and other outer membrane components (Riezu-Boj *et al.*, 1986). Thus, the HS extract contains LPS determinants specific for *B. ovis*, but also additional antigenic epitopes, some of them being shared by rough and smooth *Brucella* (Santos *et al.*, 1984). Such epitopes account for the cross-reactivity that is sometimes observed with the HS method and sera of sheep infected with *B. melitensis* or vaccinated with *B. melitensis* Rev.1 (Riezu-Boj *et al.*, 1986). The HS extract is the most widely and currently used for the serological diagnosis of *B. ovis* infection. Its water solubility and high content in relevant cell surface epitopes explain its good performance in *B. ovis* serological tests. However, in areas where *B. melitensis* infection also exists or vaccination with *B. melitensis* Rev.1 is applied in sheep, the specificity of the diagnosis with regard to *B. ovis* has to be carefully interpreted taking into account the results of serological tests for smooth *Brucella* (Blasco, 2010).

Solid basal non-selective media described in Section B.1.3 are satisfactory for the growth of *B. ovis* REO 198.

2.1.1. Preparation of HS antigen

i) Exponentially grow the REO 198 *B. ovis* strain in one of the following ways: for 48 hours in trypticase–soy broth flasks in an orbital incubator at 37 C ± 2 C and 150 rpm; or in Roux bottles of trypticase–soy agar, or other suitable medium; or in a batch-type fermenter as described for *B. abortus*. Addition of 5% serum to the medium is optional as the REO 198 *B. ovis* strain is serum-independent.

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

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

iv) After cooling, the suspension is centrifuged (15,000 g, 5°C ± 3°C, 15 minutes) and the supernatant fluid is filtered and dialysed against purified water using 100 times the volume of the suspension, at 4°C; the water should be changed three times over a minimum of 2 days.

v) The dialysed fluid can be ultracentrifuged (100,000 g, 4°C, 6–8 hours), and the sediment is resuspended in a small amount of purified water and freeze-dried. When produced to be

---

1   Obtainable from the WOAH Reference Laboratory for brucellosis in the United Kingdom: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3.
2   Obtainable from the WOAH Reference Laboratory for brucellosis in France.

used in the CFT, the addition of control process serum replacement II (CPSRII) prior to freeze-drying may assist in stability and anti-complementary activity.

HS is then resuspended either in purified water (for use in the AGID test), veronal buffered saline (for use in the CFT), or carbonate/bicarbonate buffer (for use in the I-ELISA) and titrated accordingly.

If it is to be used in the AGID test, the resuspended HS may be kept at 5°C ± 3°C adding optionally 0.5% phenol as preservative. Freezing and thawing of antigen suspensions should be avoided (Diaz & Bosseray, 1973).

The CFT antigen should be standardised against the ISaBoS to give 50% fixation at a 1/100 serum dilution. It must be emphasised that each CFT antigen batch must be titrated with the CFT procedure that is to be followed for the routine test. Therefore before using a CFT antigen (commercial or in-house) in a particular CFT procedure, the laboratory should ensure that the antigen titre has been established with the same CFT procedure.

In the absence of well-established standardisation rules, the I-ELISA and AGID antigens should be titrated against a set of appropriate positive and negative sera.

2.1.2. Standardisation of the I-ELISA

The following criteria for standardisation of the I-ELISA have been used in a recent work in which the I-ELISA has been validated in comparison with the CFT (Praud *et al.,* 2012):

i)   A 1/64 pre-dilution of the ISaBoS made up in a negative serum (or in a negative pool of sera) must give a positive reaction;

ii) A 1/256 pre-dilution of the ISaBoS made up in a negative serum (or in a negative pool of sera) must give a negative reaction.

These criteria still need to be validated through an international ring-trial.

In any case, I-ELISA commercial or in-house kits must have been validated according to Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals.*

2.2. Complement fixation test

There is no standardised method for the CFT and the use of an International Standard serum is therefore recommended (see Section B.2.2.2). The test is most conveniently carried out using the microtitration method. Some evidence shows that cold fixation is more sensitive than warm fixation (Ris *et al.*, 1984), but that it is less specific. Anticomplementary reactions, common with sheep serum, are, however, more frequent with cold fixation.

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

Barbital (veronal) buffered saline (VBS) is the standard diluent for the CFT. This is prepared from tablets available commercially, otherwise it may be prepared according to the formula described elsewhere (see Chapter 3.1.4). The test sera should be inactivated for 30 minutes in a water bath at 60–63°C, and then diluted (doubling dilutions) in VBS. The stock solution of HS antigen (2.5–20 mg/ml in VBS) is diluted in VBS as previously determined by titration (checkerboard titration). Usually, only one serum dilution is tested (generally 1/10).

2.2.1. Test procedure

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

i) Volumes of 25 µl of diluted inactivated test serum are placed in the well of the first and second rows. The first row is an anti–complementary control for each serum. Volumes of 25 µl of VBS are added to the wells of the first row (anti–complementary controls) to compensate for lack of antigen. Volumes of 25 µl of VBS 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 wells in the first row.

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

iv) Control wells are set up to contain 75 µl total volume in each case; the wells contain

a) diluent only,

b) complement + diluent,

c) antigen + complement + diluent.

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 ± 2°C for 30 minutes or at 5°C ± 3°C overnight, and a volume (25 or 50 µl according to the techniques) of sensitised SRBCs is added to each well. The plates are reincubated at 37°C ± 2°C for 30 minutes.

vi) The results are read after the plates have been centrifuged at 1000 g for 10 minutes at 5°C ± 3°C or left to stand at 5°C ± 3°C for 2–3 hours at least to allow unlysed cells to settle. The degree of haemolysis is compared with standards corresponding to 0, 25, 50, 75 and 100% lysis. The titre of the serum under test is the highest dilution in which there is 50% or less haemolysis.

2.2.2. Standardisation of the results of the complement fixation test

There is a unit system that is based on the International Standard for anti-*Brucella* ovis Serum (ISaBoS or International Standard 1985 [see footnote 3]). This serum contains 1000 ICFTU 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: 1000/200 × titre of test serum = number of ICFTU (International CFT units) of antibody in the test serum per ml. It is recommended that any country using the CFT on a national scale should obtain agreement among the different laboratories performing the test by the same method, to allow the same level of sensitivity and specificity to be obtained against an adequate panel of sera from *B. ovis* culture positive and *Brucella*-free sheep. Results should always be expressed in ICFTU, calculated in relation to those obtained in a parallel titration with a standard serum, which itself may be calibrated against the International Standard.

2.2.3. Interpretation of the results

Sera giving a titre equivalent to 50 ICFTU/ml or more are considered to be positive.

2.3. Enzyme-linked immunosorbent assay

Several variations of this assay have been proposed. The assay described here is an indirect I-ELISA using ABTS (2,2'-azino-bis-[3-ethylbenzothiazoline-6-sulphonic acid]) as chromogen, but other procedures are also suitable, and several commercial kits are now available.

Tests are performed on 96-well flat-bottomed ELISA plates.

Reagent and serum dilutions are made in PBS, pH 7.2 (± 0.2), with the addition of 0.05% Tween 20 (PBST).

Antigen dilutions are made for adsorption in a carbonate/bicarbonate buffer (pH 9.6 ± 0.2). Plates are washed after antigen coating and between incubations, where appropriate, usually with PBST (see below). The antigen (HS) and conjugate are checkerboard titrated, and dilutions are selected to give the best discriminating ratio between negative and positive standard sera. Secondary antibodies (e.g. anti-ovine IgG [H+L chains]) are usually conjugated to horseradish peroxidase (HRPO), although other enzymes or conjugates (such as recombinant Protein G/HRPO) can be used. A monoclonal antibody to bovine IgG$_1$–HRPO conjugate has also been found to be suitable for use in the I-ELISA (Vigliocco *et al.,* 1997). If a peroxidase conjugate is used, the chromogen, usually ABTS, is diluted in a substrate buffer (composed of sodium citrate and citric acid, see below)[3]. The substrate, hydrogen peroxide (H_2O_2), is added to this, and the plates are incubated for 15–30 min at room temperature (22°C ± 4°C). The reaction may be stopped with 1 mM sodium azide or other reagents, and the colour change is read at 405–414 nm (for further details see Chapter 3.1.4).

The antigen used in the I-ELISA is the HS in stock solution at 1 mg/ml in coating buffer, titrated in a checkerboard titration manner, with different dilutions of antigen, conjugate and substrate, against a standard serum or against serial dilutions of a panel of sera from *B. ovis* culture positive and *Brucella*-free sheep to determine the most sensitive and specific working concentration. Other antigens have been reported in the literature, in particular R-LPS (Nielsen *et al.,* 2004), but its extraction is cumbersome and dangerous, and it has no particular advantage compared with the HS that is also used in CFT and AGID.

A positive and a negative control are included in each plate. OD ranges to be obtained with these two controls must be established to define the criteria for validating each plate results. The positive control OD is the one to which each test serum OD is compared to establish the final result (negative or positive).

An additional positive serum (internal control) must be included in each plate to validate the repeatability of the test from plate to plate and from day to day.

2.3.1. Test procedure (example)

i) Microtitre plates of good quality polystyrene (this is important to obtain consistent results since there are differences in adsorption among different brands) are coated by the addition of 100 µl to each well of a predetermined antigen dilution in the adsorption buffer:

Adsorption buffer (0.06 M carbonate–bicarbonate buffer, pH 9.6 ± 0.2):

a) Solution A: 0.84 g $NaHCO_3$ in 10 ml purified water.

b) Solution B: 1.06 g Na_2CO_3 in 10 ml purified water.

Mix 4.53 ml of A with 1.82 ml of B and complete with purified water to 100 ml.

Sealed plates are incubated at 37°C ± 2°C overnight, preferably. Plates are then washed four times with the washing buffer to remove unbound antigen and dried by tapping firmly upside down on an absorbent paper.

Washing buffer (0.01 M PBS, pH 7.2 ± 0.2, and containing 0.05% Tween 20):

a) Stock solution:

Solution A: Na_2HPO_4: 10.96 g in 150 ml purified water

Solution B: NaH_2PO_4 (H_2O): 3.15 g in 150 ml purified water (3.5 g in 150 ml purified water if using NaH_2PO_4 2(H_2O)

---

3   TMB (3,3',5,5'-tetramethylbenzidine) is also a popular chromogenic substrate for HRP detection in ELISA and is available in several formats. It is not carcinogenic.

b) Mix A and B then complete to 400 ml with purified water.

c) Washing Buffer (PBST): 40 ml of Stock solution + 8.5 g NaCl and complete to 1000 ml with purified water, adding 0.05% Tween 20.

   The coated and washed plates can be used immediately or dried and stored at 5°C ± 3°C (the stability in these conditions is usually adequate for at least 1 month). Most of HS batches perform properly when used at working concentrations of 2.5–15 µg/ml in adsorption buffer.

ii) *Sera*: Dilute test and positive and negative control serum samples (1/100 -1/200 are usually the optimal working dilutions, prepared by the addition of 10 µl of serum to 1–2 ml PBST, respectively). These working serum dilutions are usually the optimal when using either polyclonal or monoclonal anti-IgG conjugates. However, lower working dilutions (usually 1/50) are the optimal when using the protein G-HRPO conjugates (Marin *et al.,* 1998). Add 100 µl/well volumes of samples in duplicate to the microtitre plates. The plates are covered or sealed, incubated at 37°C ± 2°C for 40–60 minutes, and washed three times with the PBST washing buffer.

iii) *Conjugate*: The optimal working dilution of titrated conjugate (the most widely used are the protein G or polyclonal rabbit anti-sheep IgG (H+L), both coupled to HRPO) in PBST is added (100 µl) to the wells, and the plates covered and then incubated for 40–60 minutes at 37°C ± 2°C After incubation, the plates are washed again three times with PBST.

iv) *Substrate*: There are several possibilities but the substrate most widely used[4] is usually composed by a 0.1% solution (w/v) of ABTS (2-2'-azinobis 3- ethylbenzthiazoline sulfonic acid, diammonium salt) in citrate buffer containing 0.004% H_2O_2):

   Citrate buffer (0.05 M, pH 4 ± 0.2):

a) Solution A: 22.97 g citric acid ($C_6H_8O_7.H_2O$) in 1000 ml purified water.

b) Solution B: 29.41 g sodium citrate ($Na_3C_6H_5O_7.2H_2O$) in 1000 ml purified water.

   Mix 660 ml of A with 470 ml of B and complete to 2000 ml with purified water. Add then a 0.004% of good grade and fresh H_2O_2.

   The substrate solution is added (100 µl/well) and the plates incubated for 15–30 minutes at room temperature with continuous shaking)

v) *Reading and interpreting the results:* Absorbance is read automatically in a spectrophotometer at 405–414 nm. Mean absorbance values may be expressed as percentages of the mean absorbance values of the positive control or, preferably, transformed into I-ELISA units calculated either manually or by using a computer and a curve-fitting program from a standard curve constructed with the series of positive control dilution results. Duplicate readings of each serum should be similar. In case of significant discrepancies, the particular serum should be retested. Before calculating the final results, each plate must be validated taking into account the OD values obtained for the positive and negative controls as well as the transformed OD of the internal control according to pre-established expected ranges of values.

The cut-off threshold to differentiate the positive and negative results should be properly established using the appropriate validation techniques (see Chapter 1.1.6) and avoiding, if possible, cut-off thresholds resulting in inconclusive results. The ISaBoS or the corresponding secondary or national standards should be used to verify or calibrate the particular test method in question as mentioned above

---

4   TMB (3,3',5,5'-tetramethylbenzidine) is also a popular chromogenic substrate for HRP detection in ELISA and is available in several formats. It is not carcinogenic.

2.4. Agar gel immunodiffusion test

The AGID test (Blasco, 1990) uses the following reagents: Good grade Noble agar or agarose, sodium chloride (NaCl), and borate buffer (prepared with boric acid [12.4 g]; potassium chloride [14.5 g]; purified water [1600 ml]; adjusted to pH 8.3 ± 0,02 with 0.2 M NaOH solution and made up to 2000 ml with purified water).

2.4.1. Agar gel preparation

Dissolve 1 g of agarose (or Noble agar) and 10 g of NaCl in 100 ml of borate buffer (by boiling while stirring continuously).

On a flat surface, cover clean glass slides with the necessary amount of molten gel to form a bed of 2.5 mm depth (3.5 ml approximately for standard microslides).

After the gel has solidified (15–20 minutes), wells are cut in it using a gel puncher.

The wells should be 3 mm in diameter and 3 mm apart, and should be arranged in a hexagonal pattern around a central well that is also 3 mm in diameter.

The test can be adapted to Petri dishes and other patterns.

2.4.2. Test procedure

Sera to be examined are placed in alternate wells separated by a control positive serum (infection proved by bacteriology), with the antigen at its optimum concentration in the central well.

The results are read after incubation for 24 and 48 hours at room temperature in a humid chamber.

A positive reaction is evidenced by a clearly defined precipitin line between the central well and the wells of the test sera that gives total or partial identity with that of the positive controls.

Precipitin lines not giving total identity may also appear and correspond usually to minor antigenic components of HS extracts (antibodies to these components can also be common in infections due to *B. melitensis* or in case of vaccination with Rev.1). These reactions should also be considered as positive. Before a definitive reading, it is important to wash the slides for 1 h in a 5% sodium citrate solution in purified water to clean unspecific precipitin lines.

The HS (2.5–20 mg/ml) diluted in purified water (optionally containing 0.5% phenol as a preservative) is the most widely used antigen in the AGID test (the preserved antigen can be stored refrigerated for at least 1 month). Dilutions of antigen are tested with a panel of 20–30 sera from rams naturally infected with *B. ovis* and with a panel of *Brucella*-free sheep. The optimum working concentration of antigen is the one giving the clearest precipitation lines with all control sera from *B. ovis*-infected rams, resulting simultaneously negative with the sera from *Brucella*-free animals.

Comparative studies have shown that the I-ELISA has a better sensitivity than either the AGID test or the CFT (Blasco, 2010; Gall *et al.,* 2003; Praud *et al.,* 2012; Ris *et al.,* 1984). However, due to the existence of some I-ELISA-negative but AGID (or CFT) positive sera and vice versa, the parallel combination of the AGID (or CFT) and I-ELISA results usually in optimal sensitivity and may be helpful in eradication programmes in infected zones or flocks (Blasco, 2010; Praud *et al.,* 2012).

Moreover, the CFT has other important disadvantages such as complexity, obligatory serum inactivation, anti-complementary activity of some sera, the difficulty of performing it with haemolysed sera, and prozone phenomena. Because of their sensitivity, simplicity and easy interpretation, both the I-ELISA and AGID test are therefore preferred for surveillance in free or almost-free zones.

Little is known about the existence of false positive results in *B. ovis* serological tests as a consequence of infections due to bacteria showing cross-reacting epitopes with *B. ovis*. The foot rot agent (*Dichelobacter nodosus*) has been described as responsible for serological cross-reactions with *B. ovis*, but the extent and practical consequences of this cross-reactivity in *B. ovis* diagnostic tests is not well understood. In addition, *Arcanobacterium pyogenes* and *Corynebacterium ovis*, whose soluble extracts cross-react with sera from *B. ovis* infected rams, have been isolated from several lymph nodes of rams giving strong positive responses in both *B. ovis* AGID and I-ELISA tests (Blasco, 2010; Blasco & Moriyon, unpublished results).

C. REQUIREMENTS FOR VACCINES

As both rams and ewes can play a role in the transmission of infection (Blasco, 2010; Grilló *et al.,* 1999), vaccination of both rams and ewes is probably the most economical and practical means for medium-term control of *B. ovis* in areas with a high prevalence of infection. For long-term control, consideration should be given to the effect of vaccination on serological testing, and the potential complication of the implementation of *B. ovis*-free accreditation programmes.

There is no specific vaccine for *B. ovis*, however live *B. melitensis* strain Rev.1 (described in Chapter 3.1.4 including the quality requirements) is also suitable to stimulate immunity against *B. ovis* infection (Blasco, 1990). A single standard dose (10^9 colony-forming units) of Rev.1 administered subcutaneously (in a 1 ml volume) or, better, conjunctivally (in a 25–30 μl volume), to 3–5 month-old animals confer adequate immunity against *B. ovis*. Conjunctival vaccination has the advantage of minimising the intense and long-lasting serological response evoked by subcutaneous vaccination, thereby improving the specificity of serological tests (Blasco, 1990), and facilitating the interpretation of serological results after vaccination. When used in both young and adult males, the safety of the Rev.1 vaccine has been shown to be adequate and side-effects appear to be very rare (Marin *et al.,* 1990; Muñoz *et al.,* 2008). Therefore, in countries with extensive management and high levels of prevalence, it would be advisable to vaccinate both young and healthy adult animals (see Chapter 3.1.4). In countries affected by *B. ovis* but free of *B. melitensis,* before using the Rev.1 vaccine account should be taken of possible serological interferences and the conjunctival route should be preferred to minimise this problem.

The *B. abortus* RB51 vaccine has not been proven successful against *B. ovis* in sheep (Jiménez De Bagües *et al.,* 1995), and despite the promising results obtained with new generation subcellular vaccines (Cassataro *et al.,* 2007; Da Costa Martins *et al.,* 2010; Muñoz *et al.,* 2006), none has yet been licensed for field use.

REFERENCES

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

BLASCO J.M. (1990). *Brucella ovis*. *In:* Animal Brucellosis, Nielsen K. & Duncan J.R., eds. CRC Press, Boca Raton, Florida, USA, 351–378.

BLASCO J.M. (2010). *Brucella ovis* infection. *In:* Infectious and Parasitic Diseases of Livestock, Lefèvre P.C., Blancou J., Chermette R. & Uilenberg G., eds. Lavoisier, Paris, France. Vol. 2:1047–1063.

BULGIN M.S. & ANDERSON B.C. (1983). Association of sexual experience with isolation of various bacteria in cases of ovine epididymitis. *J. Am. Vet. Med. Assoc.*, **182**, 372–374.

CASSATARO J., PASQUEVICH K.A., ESTEIN S.M., LAPLAGNE D.A., ZWERDLING A., DE LA BARRERA S., BOWDEN R., FOSSATI C.A., GIAMBARTOLOMEI G.H. & GOLDBAUM F.A. (2007). A DNA vaccine coding for the chimera BLSOmp31 induced a better degree of protection against *B. ovis* and a similar degree of protection against *B. melitensis* than Rev.1 vaccination. *Vaccine*, **25**, 5958–5967.

DA COSTA MARTINS R., IRACHE J.M., BLASCO J.M., MUÑOZ M.P, MARÍN C.M., JESÚS GRILLO M., JESÚS DE MIGUEL M., BARBERÁN M. & GAMAZO C. (2010). Evaluation of particulate acellular vaccines against *Brucella ovis* infection in rams. *Vaccine*, **28**, 3038–3046.

DE MIGUEL M.J., MARÍN C.M., MUÑOZ P.M., DIESTE L., GRILLÓ M.J. & BLASCO J.M. (2011). Development of a selective culture medium for primary isolation of the main *Brucella* species. *J. Clin. Microbiol.*, **49**, 1458–1463.

DIAZ R. & BOSSERAY N. (1973). Identification d'un composé antigénique spécifique de la phase rugueuse (R) des *Brucella*. *Ann. Rech. Vet.,* **4,** 283–292.

ESTEIN S.M., BALDI P.C. & BOWDEN R.A. (2002). Comparison of serological tests based on outer membrane or internal antigens for detecting antibodies to *Brucella ovis* in infected flocks. *J. Vet. Diagn. Invest.*, **14**, 407-411.

GALL D., NIELSEN K., VIGLIOCCO A., SMITH P., PEREZ B., ROJAS X. & ROBLES C. (2003). Evaluation of an indirect enzyme-linked immunoassay for presumptive serodiagnosis of *Brucella ovis* in sheep. *Small Rumin. Res.*, **48**, 173–179.

GARCÍA-PASTOR L., BLASCO J.M. & BARBERÁN M. (2009). Pasteurellosis as a cause of genital lesions in rams. A descriptive study. *Small Rumin. Res.*, **87**, 111–115.

GRILLÓ M.J., MARÍN C., BARBERÁN M. & BLASCO J.M (1999). Experimental *Brucella ovis* infection in pregnant ewes. *Vet. Rec.*, **144**, 555–558.

JIMÉNEZ DE BAGÜES M.P., BARBERÁN M., MARÍN C.M. & BLASCO J.M. (1995). The *Brucella abortus* RB51 vaccine does not confer protection against *Brucella ovis* in rams. *Vaccine*, **13**, 301–304.

LIVINGSTONE C.W. & HARDY W.T. (1964). Isolation of *Actinobacillus seminis* from ovine epididymitis. *Am. J. Vet. Res.,* **25**, 660–663.

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

MARÍN C.M., ALONSO-URMENETA B., MORIYÓN I., PEREZ S. & BLASCO J.M. (1998). Comparison of polyclonal, monoclonal and protein G peroxidase conjugates in an enzyme-linked immunosorbent assay for the diagnosis of *Brucella ovis* in sheep. *Vet. Rec.,* **143**, 390–394.

MARÍN C.M., BARBERÁN M., JIMÉNEZ DE BAGÜES M.P. & BLASCO J.M. (1990). Comparison of subcutaneous and conjunctival routes of Rev. 1 vaccination for the prophylaxis of *Brucella ovis* infection in rams. *Res. Vet. Sci.,* **48**, 209–215.

MUÑOZ P., DE MIGUEL M.J., GRILLÓ M.J., MARÍN C.M., BARBERÁN M. & BLASCO J.M. (2008). Immunopathological responses and kinetics of *B. melitensis* Rev 1 infection after subcutaneous or conjunctival vaccination in rams. *Vaccine*, **26**, 2562–2569.

MUÑOZ P.M., ESTEBAN M., MARÍN, C.M., DE MIGUEL M.J., GRILLÓ M.J., BARBERÁN M., IRACHE J.M., BLASCO J.M. & GAMAZO C. (2006). *Brucella* outer membrane complex-loaded microparticles as a vaccine against *Brucella ovis* in rams. *Vaccine*, **24**, 1897–1905.

MYERS D.M., JONES L.M. & VARELA-DIAZ V. (1972). Studies of antigens for complement fixation and gel diffusion tests in the diagnosis of infections caused by *Brucella ovis* and other *Brucella. Appl. Microbiol.,* **23**, 894–902.

NIELSEN K., SMITH P., CONDE S., DRAGHI DE BENITEZ G., GALL D., HALBERT G., KENNY K., MASSENGILL C., MUENKS Q., ROJAS X, PEREZ B., SAMARTINO L., SILVA P., TOLLERSRUD T. & JOLLEY M. (2004). Rough lipopolysaccharide of *Brucella abortus* RB51 as a common antigen for serological detection of *B. ovis, B. canis*, and *B. abortus* RB51 exposure using indirect enzyme immunoassay and fluorescence polarization assay. *J. Immunoassay Immunochem.*, **25**, 171–182.

PRAUD A., CHAMPION J.L., CORDE Y., DRAPEAU A., MEYER L. & GARIN-BASTUJI B. (2012). Assessment of the diagnostic sensitivity and specificity of an indirect ELISA kit for the diagnosis of *Brucella ovis* infection in rams. *BMC Vet. Res.*, **8**:68.

RIDLER A.L., WEST D.M. & COLETT M.G. (2012). Pathology of *Brucella ovis* infection in red deer stags (*Cervus elaphus*). *N. Z. Vet. J.*, **60**, 146–149.

RIEZU-BOJ J.I., MORIYÓN I., BLASCO J.M., MARÍN C.M. & DIAZ R. (1986). Comparison of lipopolysaccharide and outer membrane protein-lipopolysaccharide extracts in an enzyme-linked immunosorbent assay for the diagnosis of *Brucella ovis* infection. *J. Clin. Microbiol.,* **23**, 938–942.

RIS D.R., HAMEL K.L. & LONG D.L. (1984). Comparison of an enzyme-linked immunospecific assay (ELISA) with the cold complement fixation test for the serodiagnosis of *Brucella ovis* infection. *N.Z. Vet. J.,* **32**, 18–20.

SANTOS J.M., VERSTREATE D.R., PERERA V.Y. & WINTER A.J. (1984). Outer membrane proteins from rough strains of four *Brucella* species. *Infect. Immun.,* **46**, 188–194.

VIGLIOCCO A.M., SILVA PAULO P.S., MESTRE J., BRIONES G.C., DRAGHI G., TOSSI M. & NIELSEN K. (1997). Development and validation of an indirect enzyme immunoassay for detection of ovine antibody to *Brucella ovis. Vet. Microbiol.*, **54**, 357–368.

XAVIER M.N., SILVA T.M.A., COSTA E.A., PAIXAO T.A., MOUSTACAS V.S., CARVALHO C.A. Jr., SANT'ANNA F.M., ROBLES C.A., GOUVEIA A.M.G., LAGE A.P., TSOLIS R.M. & SANTOS R.L. (2010). Development and evaluation of a species-specific PCR assay for the detection of *Brucella ovis* infection in rams. *Vet. Microbiol.*, **145**, 158–164.

*

* *

NB: There are WOAH Reference Laboratories for ovine epididymitis (*Brucella ovis*)
(please consult the WOAH Web site:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for ovine epididymitis (*Brucella ovis*)

NB: FIRST ADOPTED IN 1991 AS BRUCELLOSIS IN SHEEP, GOATS AND SWINE; CHAPTER FIRST ADOPTED WITH CURRENT TITLE IN 1996. MOST RECENT UPDATES ADOPTED IN 2015.