

CHAPTER 2.7.9.

OVINE EPIDIDYMITIS (*Brucella ovis*)

SUMMARY

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

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

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

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

A. INTRODUCTION

Brucella ovis causes a genital infection of ovine livestock manifested by epididymitis, infrequent abortions, and increased lamb mortality. Passive venereal transmission via the ewe appears to be a frequent route of infection, but ram-to-ram transmission is also common¹ (2). Infected ewes may excrete *B. ovis* in vaginal discharges and

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

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

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

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

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

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

a) Collection of samples

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

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

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

b) Staining methods

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

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

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

c) Culture

The best direct method of diagnosis is bacteriological isolation on adequate culture media. Semen, vaginal swabs, or milk samples can be smeared directly on to plates with adequate culture media and incubated at 37°C in an atmosphere of 5–10% CO₂. Tissues should be macerated and ground in a small amount of sterile saline or phosphate buffered saline (PBS) with a stomacher or blender, before plating.

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

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

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

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

Antibiotic solution (prepared daily): colistin, nystatin and vancomycin are suspended in a mixture of methanol/water (1/1); nitrofurantoin is suspended in 1 ml of a 0.1 M NaOH sterile solution. For amphotericin B, it is recommended to prepare a stock solution of 10 mg/ml amphotericin B with 10 mg dissolved first in 1 ml sterile dimethyl sulphoxide (C₂H₆OS, for analysis; ACS) and then added to 9 ml of PBS (10 mM, pH 7.2). Any stock solution remaining can be stored some days at 4°C. All antibiotic solutions must be filtrated through 0.22 µm filters before addition to the culture medium.

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

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

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

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

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

d) Identification and typing

Brucella ovis colonies are not haemolytic. They are circular, convex, have unbroken edges, are always of the rough type when examined by oblique illumination, and test positive in the acriflavine test (1, 7). For growth, *B. ovis* needs an atmosphere of 5–10% CO₂. It lacks urease activity, fails to reduce nitrate to nitrite, is catalase positive and oxidase negative. It does not produce H₂S and, although it does not grow in the presence of methyl violet, it usually grows in the presence of standard concentrations of basic fuchsin and thionin. The cultures are not lysed by *Brucella*-phages of the Tbilissi (Tb), Weybridge (Wb) and Izatnagar (Iz) groups at the routine test dilution (RTD) or 10⁴ RTD, while they are lysed by phage R/C (1, 7). Most laboratories are not equipped for a complete identification, and a practical schedule for presumptive identification is needed. Most *B. ovis* isolates can be correctly identified on the basis of growth characteristics, direct observation using obliquely reflected light, Gram or Stamp's staining, catalase, oxidase, urease and acriflavine tests. However, definitive identification should be carried out by reference laboratories with experience in identification and typing of *Brucella*.

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

2. Serological tests

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

The International Standard anti-*Brucella ovis* Serum (International Standard 1985²) is the one against which all other standards are compared and calibrated. This reference standard is available to national reference laboratories and should be used to establish secondary or national standards against which working standards can be prepared and used in the diagnostic laboratory for daily routine use.

• Antigens

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

Brucella ovis REO 198, a CO₂- and serum-independent strain, is recommended as a source of the HS antigens to be used in serological tests³. Solid media described in Section B.1.c. are satisfactory for the growth of *B. ovis* REO 198. HS antigen is prepared as follows:

- i) Exponentially grow a suitable strain of *B. ovis*, preferably aerobic and nonserum dependent, e.g. REO 198, in one of the following ways: for 48 hours in trypticase–soy broth flasks in an orbital incubator at 37°C and 150 rpm; or in Roux bottles of trypticase–soy agar, or other suitable medium, with 5% serum added (not necessary when using the REO 198 strain); or in a batch-type fermenter as described for *B. abortus*, but with the addition of 5% serum to the medium (not necessary when using the REO 198 strain).
- ii) Cells are resuspended in 0.85% saline or PBS, then washed twice in 0.85% saline (12 g of dried cells or 30 g of wet packed cells in 150 ml).
- iii) The cell suspension is then autoclaved at 120°C for 15–30 minutes.
- iv) After cooling, the suspension is centrifuged (15,000 **g**, 4°C, 15 minutes) and the supernatant fluid is filtered and dialysed against distilled water using 100 times the volume of the suspension, at 4°C; the water should be changed three times over a minimum of 2 days.
- v) The dialysed fluid can be ultracentrifuged (100,000 **g**, 4°C, 6–8 hours), and the sediment is resuspended in a small amount of distilled water and freeze-dried. When produced to be used in the CFT, the addition of control process serum replacement II (CPSRII) prior to freeze-drying may assist in stability and anti-complementary activity.

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

2 Obtainable from the OIE Reference Laboratory for Brucellosis at VLA Weybridge, Addlestone, Surrey KT15 3NB, United Kingdom.

3 Obtainable from the OIE Reference Laboratory for Brucellosis at AFSSA, 94706, Maisons-Alfort, France.

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

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

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

Several methods have been proposed for the CFT using different concentrations of fresh sheep red blood cells (SRBCs) (a 2–3% suspension is usually recommended) sensitised with an equal volume of rabbit anti-SRBC serum diluted to contain several times (usually from two to five times) the minimum concentration required to produce 100% lysis of SRBCs in the presence of a titrated solution of guinea-pig complement. The latter is independently titrated (in the presence or absence of antigen according to the method) to determine the amount of complement required to produce either 50% or 100% lysis of sensitised SRBCs in a unit volume of a standardised suspension; these are defined as the 50% or 100% haemolytic unit of complement (C'H₅₀ or C'H₁₀₀), respectively. It is generally recommended to titrate the complement before each set of tests, a macromethod being preferred for an optimal determination of C'H₅₀. Usually, 1.25–2 C'H₁₀₀ or 5–6 C'H₅₀ are used in the test.

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

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

- i) Volumes of 25 µl of diluted inactivated test serum are placed in the well of the first and second rows. Volumes of 25 µl of CFT buffer are added to all wells except those of the first row. Serial doubling dilutions are then made by transferring 25 µl volumes of serum from the second row onwards.
- ii) Volumes of 25 µl of antigen, diluted to working strength, are added to each well except wells in the first row.
- iii) Volumes of 25 µl of complement, diluted to the number of units required, are added to each well.
- iv) Control wells containing diluent only, complement + diluent, antigen + complement + diluent, are set up to contain 75 µl total volume in each case. A control serum that gives a minimum positive reaction should be tested in each set of tests to verify the sensitivity of test conditions.
- v) The plates are incubated at 37°C for 30 minutes or at 4°C overnight, and a volume (25 or 50 µl according to the techniques) of sensitised SRBCs is added to each well. The plates are reincubated at 37°C for 30 minutes.
- vi) The results are read after the plates have been centrifuged at 1000 *g* for 10 minutes at 4°C or left to stand at 4°C for 2–3 hours to allow unlysed cells to settle. The degree of haemolysis is compared with standards corresponding to 0, 25, 50, 75 and 100% lysis. The titre of the serum under test is the highest dilution in which there is 50% or less haemolysis.

• Standardisation of the results of the complement fixation test

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

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

b) Agar gel immunodiffusion test

The AGID test (2) uses the following reagents: Good grade Noble agar or agarose, sodium chloride (NaCl), and borate buffer (prepared with boric acid [12.4 g]; potassium chloride [14.5 g]; distilled water [1600 ml]; adjusted to pH 8.3 with 0.2 M NaOH solution and made up to 2000 ml with distilled water).

To prepare the gels, dissolve 1 g of agarose (or Noble agar), 10 g of NaCl and 100 ml of borate buffer by boiling while stirring continuously. On a flat surface, cover clean glass slides with the necessary amount of molten gel to form a bed of 2.5 mm depth (3.5 ml approximately for standard microslides). After the gel has solidified (15–20 minutes), wells are cut in it using a gel puncher. The wells should be 3 mm in diameter and 3 mm apart, and should be arranged in a hexagonal pattern around a central well that is also 3 mm in diameter. The test can be adapted to Petri dishes and other patterns.

Sera to be examined are placed in alternate wells separated by a control positive serum (infection proved by bacteriology), with the antigen at its optimum concentration in the central well. The results are read after incubation for 24 and 48 hours at room temperature in a humid chamber. A positive reaction is a clearly defined precipitin line between the central well and the wells of the test sera that gives total or partial identity with that of the positive controls. Precipitin lines not giving total identity may also appear and correspond to minor antigenic components of HS extracts (antibodies to these components can also be common in infections due to *B. melitensis*). These reactions should also be considered to be positive. Before a definitive reading, it is important to wash the slides for 1 hour in a 5% sodium citrate water solution to clean unspecific precipitin lines.

The HS (2.5–20 mg/ml) diluted in distilled water (optionally containing 0.5% phenol as a preservative) is the antigen used in the AGID test (the preserved antigen can be stored at 4°C for at least 1 month). Dilutions of antigen are tested with a panel of 20–30 sera from rams naturally infected with *B. ovis* and with a panel of *Brucella*-free sheep. The optimum concentration of antigen is that giving the clearest precipitation line with all the sera from *B. ovis*-infected rams being negative with the sera from *Brucella*-free sheep.

c) Enzyme-linked immunosorbent assay (the alternative test for international trade)

Several variations of this assay have been proposed. The assay described here is an indirect I-ELISA using ABTS (2,2'-azino-bis-[3-ethylbenzothiazoline-6-sulphonic acid]) as chromogen, but other procedures are also suitable. Tests are performed on 96-well flat-bottomed ELISA plates. Reagent and serum dilutions are made in PBS, pH 7.2, with the addition of 0.05% Tween 20 (PBST). Antigen dilutions are made in a carbonate/bicarbonate buffer, pH 9.6, or, alternatively, in PBS, pH 7.2. Plates are washed after antigen coating and between incubations, where appropriate, usually with PBST. The antigen (HS) and conjugate are checkerboard titrated, and dilutions are selected to give the best discriminating ratio between negative and positive standard sera. Secondary antibodies (anti-ovine IgG [H + L chains]) antibodies are usually conjugated to horseradish peroxidase (HRPO), although other enzymes or conjugates (such as recombinant protein G/HRPO) can be used. A monoclonal antibody to bovine IgG₁-HRPO conjugate has also been found to be suitable for use in the I-ELISA (26). If a peroxidase conjugate is used, the chromogen, usually ABTS, is diluted in a substrate buffer (composed of citric acid trisodium and citric acid; pH 4). The substrate, hydrogen peroxide (H₂O₂), is added to this, and the plates are incubated for 15–25 minutes at room temperature. The reaction may be stopped with 1 mM sodium azide, and the colour change is read at 405–414 nm (for further details see Chapter 2.4.3).

The antigen used in the I-ELISA is the HS in stock solution at 1 mg/ml in coating buffer, titrated in a checkerboard titration, with different dilutions of antigen, conjugate and substrate, against a standard serum or against serial dilutions of a panel of sera from *B. ovis* culture positive and *Brucella*-free sheep to determine the most sensitive and specific dilution (usually 5–10 µg/ml).

• Test procedure (example)

- i) Microtitre plates of good quality polystyrene are coated by the addition of 100 µl of a predetermined antigen dilution in carbonate buffer, pH 9.6, to each well. Plates are incubated for 2 hours at 37°C. Alternatively, the coating can be made overnight at 4°C with 100 µl/well of the predetermined antigen dilution in PBS, pH 7.2. Plates are then washed four times to remove unbound antigen and dried by tapping firmly upside down on an absorbent paper. The coated plates can be used immediately or dried and stored at 4°C (the stability in these conditions is adequate for at least 1 month).
- ii) *Sera*: Dilute test and positive and negative control serum samples 1/200 by the addition of a minimum of 10 µl of serum to 2 ml PBST. This serum dilution is usually the optimal when using both polyclonal and monoclonal conjugates. However, dilutions of 1/50 are the optimal when using the protein G-peroxidase conjugate (14) Add 100 µl/well volumes of samples in duplicate to the microtitre plates. The plates are covered, incubated at 37°C for 1 hour, and washed three times with the PBST washing buffer.

- iii) *Conjugate*: The titrated conjugate is diluted in PBST, added (100 µl) to the wells, and the plate is covered and incubated for 1 hour at 37°C. After incubation, the plates are washed again three times with PBST.
- iv) *Substrate*: The solution of ABTS in substrate buffer is added (100 µl/well) and the plates are incubated for 15–60 minutes at room temperature with continuous shaking.
- v) *Reading and interpreting the results*: Absorbance is read automatically in a spectrophotometer at 405–414 nm. Absorbance values may be expressed as percentages of the mean absorbance of the positive control or, preferably, transformed into I-ELISA units calculated either manually or by using a computer and a curve-fitting program from a standard curve constructed with the series of positive control dilution results.

The cut-off threshold should be properly established using the appropriate validation techniques (see Chapter 1.1.4 Principles of validation of diagnostic assays for infectious diseases). The International Standard for anti-*Brucella ovis* Serum or the corresponding secondary or national standards should be used to check or calibrate the particular test method in question.

Comparative studies have shown that the I-ELISA has better sensitivity than either the AGID test or the CFT (16, 21, 25, 31, 32). Due to the existence of some I-ELISA-negative and AGID-positive sera, the combination of the AGID test and I-ELISA gives optimal sensitivity (16). However, the combination of CF test and I-ELISA or CF and AGID tests does not improve the sensitivity of I-ELISA alone (16). Moreover, the CFT has other important disadvantages such as complexity, obligatory serum inactivation, anticomplementary activity of some sera, the difficulty of performing it with haemolysed sera, and prozone phenomena. Because of its sensitivity, simplicity and easy interpretation, the AGID test is very practicable for routine diagnosis in nonspecialised laboratories.

Little is known about the existence of false positive results in *B. ovis* serological tests as a consequence of infections due to bacteria showing cross-reacting epitopes with *B. ovis*. The foot rot agent (*Dichelobacter nodosus*) has been described as showing cross-reactions with *B. ovis* (29), but the extent and practical consequences of this cross-reactivity in *B. ovis* diagnostic tests is unknown⁴.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Vaccination of both rams and ewes is probably the most economical and practical means for medium-term control of *B. ovis* in areas with a high incidence of infection. For long-term control, consideration should be given to the effect of vaccination on serological testing, and *B. ovis*-free accreditation programmes have to be implemented. The live *B. melitensis* strain Rev.1 (see Chapter 2.7.2) is probably the best available vaccine for the prophylaxis of *B. ovis* infection (2). A single standard dose (10⁹ colony-forming units) of Rev.1 administered subcutaneously (in a 1 ml volume) or conjunctivally (in a 25–30 µl volume), to 3–5 month-old animals confers adequate immunity against *B. ovis*. Conjunctival vaccination has the advantage of minimising the intense and long-lasting serological response evoked by subcutaneous vaccination, thereby improving the specificity of serological tests (2). When used in both young and adult males, the safety of the Rev.1 vaccine is adequate and side-effects appear to be very rare (15, 17). Therefore, in countries with extensive management and high levels of incidence, it would be advisable to vaccinate both young and healthy adult animals. In countries affected by *B. ovis* but free of *B. melitensis*, before using the *B. melitensis* Rev.1 vaccine account should be taken of possible serological interferences and the conjunctival route should be preferred to minimise this problem. The *B. abortus* RB51 live vaccine has not proven successful against *B. ovis* in sheep (11) and no alternative vaccines are currently available.

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4 *Arcanobacterium pyogenes* and *Corynebacterium ovis*, whose soluble extracts cross-react with *B. ovis* positive control sera, have been recently isolated from several lymph nodes of rams giving strong positive responses in *B. ovis* AGID test and I-ELISA (J.M. Blasco, unpublished results).

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