AUSTRALIAN STANDARD DIAGNOSTIC TECHNIQUES FOR ANIMAL DISEASES

STANDING COMMITTEE ON AGRICULTURE AND RESOURCE MANAGEMENT

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Ovine Brucellosis

Bacteriology and Serology

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1. Introduction

Ovine brucellosis caused by *Brucella ovis* is primarily a genital disease of rams resulting in reduced fertility. Clinical disease occurs less commonly in ewes, although it is postulated that ewes play a significant role in the natural transmission of the disease between rams. In rams, ovine brucellosis is manifested by unilateral or, occasionally, bilateral epididymitis most commonly affecting the cauda epididymidis. In ewes, the usual clinical signs are reduced lamb drop with extension of the lambing period, poor neonatal lamb viability and infrequent abortions.

2. Diagnosis

The diagnosis of ovine brucellosis is based on:
(a) clinical evidence of disease;
(b) isolation of *B. ovis* from semen or tissues;
(c) serological evidence of infection; and
(d) histopathological examination of tissues from infected sheep.

3. Bacteriology

B. ovis is a non-motile, non-encapsulated, Gram negative, coccobacillus or short rod, usually occurring singly. Individual organisms are 0.5–0.6 µm x 0.6–1.5 µm and are acid fast when stained by the Modified Ziehl-Neelsen (MZN) technique. The organism is catalase positive and oxidase negative. The type strain is 63/290 (ATCC 25840; NCTC 10512).

Serum or blood enriched medium is required for optimal growth *in vitro*. Growth occurs in an atmosphere of 10% carbon dioxide in air but is best in a microaerophilic atmosphere. There is usually no growth of primary isolates under aerobic conditions although sporadic occurrences of aerobic strains have been reported. On solid culture medium, 72 hour colonies are 1–2 mm diameter, rough phase, round, shiny and convex.

B. ovis can be isolated from semen or the reproductive tract and associated lymph nodes of infected rams, and from placentas and aborted foetuses.

3.1. Examination of Semen

3.1.1. Collection

Samples are collected by electro-ejaculation into sterile containers. The penis should be exteriorised to reduce contamination of the sample. This is more easily achieved with the ram in a 'sitting' position. The exteriorised penis is then grasped behind the glans penis. The chances of the penis slipping back into the prepuce are reduced if a folded tissue paper is wrapped around the penis shaft to improve grip. The ram is then placed in lateral recumbency prior to inserting the rectal probe for electrostimulation. Specimens for culture should be transported to the laboratory on ice as soon as possible after collection. The organism remains viable in semen for at least 72 hours at room temperature and survival is enhanced at 4°C.

3.1.2. Microscopic Examination

Thin smears of semen should be stained with Gram stain and by the MZN technique then examined for bacteria and leucocytes.

3.1.3. Culture

The use of a selective medium to suppress overgrowth of contaminant bacteria is recommended. Each sample should be plated onto 7% blood agar and a selective medium.

3.2. Examination of Tissues from Infected Rams

The preferred culture sites in infected rams are the cauda epididymis, ampulla and seminal gland (seminal vesicle) on each side, and inguinal lymph nodes. Selected tissues should be macerated in a Colwarth Stomacher® [A.J. Seward, UK. Agents: FSE Pty Ltd, 47–49 Overseas Rd, Noble Park, Vic. 3174. Tel. (03) 795 0077; Fax (03) 790 1900] or similar apparatus and plated onto 7% sheep blood agar and selective medium (see 3.5.1.). Examination of MZN stained smears of suspect tissue may allow rapid diagnosis but the results are not as reliable as culture.

3.3. Examination of Abortion Material

Dead lambs and placentas should be examined. The preferred culture sites in aborted lambs are placenta, abomasal contents and lung. Preparation and examination of MZN stained smears may be of assistance.

3.4. Staining Methods

3.4.1. Gram Stain

There are a number of modifications of the Gram stain suitable for demonstration of *B. ovis*. Techniques are given in most standard microbiology text books.

3.4.2. Modifled Ziehl–Neelsen Stain

3.4.2.1. Dilute basic fuchsin

Basic fuchsin	1.0 g
Absolute ethanol, C_2H_5OH, to	10 mL
5% aqueous phenol, C_6H_5OH, to	100 mL

Dilute one in five with distilled water for use.

3.4.2.2. Acetic acid decolouriser

Glacial acetic acid, CH_3CO_2H	1 mL
Distilled water to	200 mL

3.4.2.3. Counterstain

1% aqueous methylene blue or 0.5% malachite green.

3.4.2.4. Staining Method

Thin smears are very gently heat fixed, stained with dilute basic fuchsin for five minutes and decolourised with 0.5% acetic acid for not more

than 30 s. Following a wash in tap-water, smears are counterstained for 90 s, washed in tap water and allowed to dry before microscopic examination under oil immersion.

B. ovis stains red against a blue–black or green background depending on the counterstain. In pathological material the organisms are usually detected intracellularly in polymorphonuclear leucocytes. Other organisms such as *Campylobacter fetus* and *Chlamydia* sp. may stain red, but can be differentiated from *Brucella* sp. by their morphology.

3.5. Culture

3.5.1. Preparation of Selective Media

Various selective media containing a combination of antibiotics to inhibit contaminant bacteria have been described for *B. ovis*. A suitable antibiotic combination is:

Vancomycin	3 mg/L
Colistin methane sulfate	7.5 mg/L
Nystatin	12 500 units/L
Fruradantin	10 mg/L

The first three antibiotics are available in a commercial preparation (VCN Selective Supplement — SR101, Oxoid Aust.). The antibiotics may be added to 7% sheep blood agar or serum-enriched nutrient agar.

3.5.2. Inoculation and Incubation

Specimens should be inoculated onto agar plates with the minimum of delay after receipt at the laboratory. Both standard sheep blood agar and selective medium should be inoculated to ensure other pathogenic bacteria are not missed. Media should be no more than two weeks old for optimal growth of *B. ovis* on primary culture.

In most laboratories, plates are incubated at 37°C in an atmosphere of 10% carbon dioxide in air. Alternatively, plates may be incubated in a candle jar. For optimal growth, a microaerophilic atmosphere can be obtained by evacuating an anaerobic jar containing the plates to a vacuum of 10 kPa (76 mm of mercury) then filling to atmospheric pressure with carbon dioxide.

3.5.3. Identification of *Brucella ovis*

Plates are examined after three days for the presence of *B. ovis* colonies and discarded after eight days if negative. Identification can be confirmed by slide agglutination tests with an anti-*B. ovis* serum.

4. Serology

The currently accepted serological test in all states of Australia is the complement fixation (CF) test (CFT). More recently, enzyme-linked immunosorbent assays (ELISA) have been employed for ovine brucellosis. Results correlated well with the CFT with an increased sensitivity.

4.1. Complement Fixation Test

The CFT is a modification of the standard Australian CFT for *Brucella abortus* as used in the bovine brucellosis eradication campaign (Anon., 1987). Anticomplementary activity is occasionally a problem in sheep serum therefore modifications include an initial serum dilution of 1:8. In addition, the primary incubation time is extended to 60 min to increase sensitivity.

The procedure outlined here should be read in conjunction with the *B. abortus* procedure.

4.1.1. Reagents

4.1.1.1. Antigen

Variation has been observed in the performance of antigen obtained from different sources. In general *B. ovis* CF antigen supplied by CSL Ltd, 45 Poplar Rd, Parkville, Vic. 3052, Aust. Tel. (03) 389 1911, (008) 032 657; Fax (03) 389 1646) has been shown to be acceptable although titres do vary from batch to batch. This material is the soluble fraction of autoclaved preparation of *B. ovis* containing crude lipopolysaccharide.

4.1.1.2. Haemolytic system

Complement, haemolysin, veronal-buffered diluent and sheep erythrocyte preparations are as per the method for *B. abortus* .

4.1.1.3. Control sera

Both positive and negative control sera should be included on each plate. Each serum should consist of a pool of appropriate positive or negative sera.

4.1.2. Procedure

(a) A microtitre procedure is used with up to 10 test sera and a positive and negative control serum per plate.
(b) Dispense 25 µL of each serum and 50 µL diluent into the respective wells of rows A and G of a microtitre plate.
(c) Cover these rows with adhesive tape and inactivate the serum by heating for 30 min in a 60°C incubator.
(d) Add 25 µL diluent to all wells in rows A–F and row H.
(e) Make doubling dilutions, beginning with row A, down the plate to row F. Similarly, dilute row G and H. The dilution series obtained is shown in Table 1.

Table 1. Complement fixation test dilution series

Row	*Serum dilution*	*Comments*
A	1:4	Doubling dilution series
B	1:8	Doubling dilution series
C	1:16	Doubling dilution series
D	1:32	Doubling dilution series
E	1:64	Doubling dilution series
F	1:128	Doubling dilution series
G	1:4	Initial dilution purposes only
H	1:8	Anticomplementary control — no antigen

(f) Dispense 25 µL of antigen into wells in rows B–F.
(g) Add 25 µL of diluent into wells in row H (anticomplementary control).
(h) Add 25 µL of complement to wells in rows B–F and row H and gently tap the plate to mix reagents.
(i) Cover and incubate for 60 min at 37°C.
(j) After removal from the incubator, add 25 µL of 1.5% haemolysin sensitised sheep erythrocytes to wells in rows B–F and row H.
(k) Place plates on a mechanical shaker in a 37°C incubator for 30 min.
(l) Centrifuge plates or let stand for one to two hours to allow unlysed cells to settle.
(m) The titre is taken as the highest dilution of serum in which 50% or less lysis occurs.
(n) Standard controls should be run on all reagents. In addition, a positive and a negative control serum should be included on each plate and every tenth plate should consist of a range of control serum samples.

4.1.3. Interpretation

All serological tests should be interpreted with consideration of the clinical history of the flock of origin. As a guide, however, a titre greater than 1:8 is considered to be positive. Any reaction at a dilution of 1:8 must be considered to be a suspect reactor.

4.1.4. Modifications

A cold CF test, in which the primary incubation is carried out overnight at 4°C, has been widely employed. This technique gives greater sensitivity but has the drawback of reduced specificity. False positive reactions are a persistent problem.

4.2. Enzyme-linked Immunosorbent Assay

The ELISA has a number of advantages over the CFT such as lower costs, increased sensitivity and long shelf-life of reagents.

4.2.1. Reagents

All reagents are prepared using analytical reagent grade materials and GLASS DISTILLED water only. Other types of distilled water may inactivate the conjugate producing low or no colour reaction. It is suggested that all reagents be prepared as concentrated stock solutions which are conveniently diluted prior to use.

4.2.1.1. Phosphate-buffered saline + Tween 20 (PBS/Tween)

Prepare a 10 times stock solution:

NaCl (m.w. 58.4) 1.4 mol/L	81.8 g
Sodium dihydrogen phosphate, $NaH_2PO_4.2H_2O$, (m.w. 156) 30 mmol/L	4.4 g
Disodium hydrogen phosphate, Na_2HPO_4. anhydrous (m.w. 141.9) 70 mol/L	10.2 g
0.5% Tween 20	5.0 g
Glass-distilled water to	1 L

The weight of sodium phosphate salts to be used is dependant on the hydration state, i.e. anhydrous, H_2O, $2H_2O$. Dilute one plus nine with glass-distilled water and use within several days. The 10 times stock solution is resistant to bacterial and fungal growth if stored at room temperature for up to three months and at 4°C for longer periods. Crystallisation of salts may occur at this temperature but can be redissolved by warming the solution prior to use. The diluted reagent contains 140 mmol/L NaCl, 10 mmol/L sodium phosphate and 0.05% Tween 20 and should have a pH of about 7.2.

4.2.1.2. Coating buffer

Prepare a 10 times stock solution:

Sodium carbonate, Na_2CO_3, (m.w. 106) 170 mmol/L	18.0 g
Sodium hydrogen carbonate, $NaHCO_3$, (m.w. 84) 330 mmol/L	27.7 g
Glass-distilled water to	1 L

Dilute one plus nine with glass-distilled water prior to use. Store the concentrate in a tightly capped bottle to prevent entry of carbon dioxide. The reagent consists of a 50 mmol/L mixture of sodium carbonate and sodium hydrogen carbonate and has a pH of about 9.5.

4.2.1.3. Antigen

B. ovis complement fixing antigen (CSL) is used. Similar antigen preparations have been produced by a number of different laboratories and groups. A large number of batches have been tested with relatively small batch variation.

4.2.1.4. Enzyme-labelled conjugates

Horseradish peroxidase (HRPO) is the preferred enzyme label. HRPO-conjugated, anti-ovine IgG (H+L Chains) can be obtained from various commercial sources (e.g. Silenus, ICN, KPL, Dako). Superior results appear to be obtained when affinity purified conjugates are used.

Differences have been observed in the suitability of different batches of commercially prepared and 'in house' anti-ovine conjugates in detecting low levels of antibody specific for *B. ovis*. This is reflected in the shape of the standard curve produced by the dilutions of the positive control serum (Fig. 1) and the ratio of the optical density of the 1:200 dilutions of the positive and negative control sera. Reasons for this are not known but probably reflect conjugate avidity. Thus it is important that conjugate preparations be fully standardised and optimised to obtain reproducible results.

4.2.1.5. Chromogen

2,2'-[azino di(3-ethylbenz-thiazoline sulfonate)] (ABTS) is the preferred chromogen. Others have been used but do not have the combination of stability, sensitivity and overall utility exhibited by ABTS. Stock solution is prepared as follows:

ABTS (m.w. 549) 50 mmol/L	2.75 g
Glass-distilled water to	100 mL

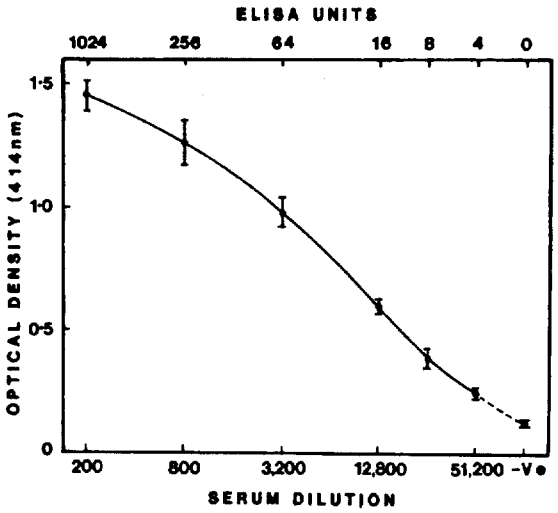

Figure 1. Titration curve of positive *B. ovis* control serum showing correlation with ELISA units. The positive control serum was tested at the dilutions indicated. The negative control serum was tested at a dilution of 1:200. Results are expressed as mean ± s.d.

Aliquots (20 mL) are stored either at room temperature (stable for at least one month) or 4°C (stable for at least 12 months).

4.2.1.6. Substrate

Prepare stock solution daily as follows.

Hydrogen peroxide, H_2O_2, (35% w/v)	126 µL
Glass-distilled water to	10 mL

4.2.1.7. Substrate buffer

Stock solution is prepared as follows:

Trisodium citrate, $Na_3C_6H_5O_7.2H_2O$, (m.w. 294) 0.023 mol/L	6.8 g
Citric acid, $C_6H_8O_7.H_2O$, (m.w. 210) 0.027 mol/L	5.7 g
Glass-distilled water to	1 L

Dispense in 100 mL aliquots and autoclave at 121°C for 15 min. This solution is stable for at least 12 months in sealed bottles.

4.2.1.8. Working substrate solution

Substrate buffer	50 volumes
ABTS solution	1 volume
H_2O_2 solution	1 volume

This solution is stable to light and air and useable for up to several days without auto-oxidising. As the reaction of peroxidase is self limiting after about one hour at room temperature, and the reagent is stable to auto-oxidation, no stopping solution is required and colour development can be measured at 414 nm (or similar) at this time.

4.2.1.9. Control sera

Positive and negative control sera are stored at -20°C with 0.02% sodium azide (NaN_3) added as a preservative. Aliquots are removed for test purposes and kept at 4°C for no longer than two months. Limited quantities of the standard positive control serum used in the development of this test are available from Regional Veterinary Laboratory, PO Box 388, Benalla, Vic. 3672, Australia.Tel. (057) 622 933; Fax (057) 623 953.

4.2.1.10. Microtitre Plates

No significant differences are observed in the performance of the test with the numerous brands of 'normal' microtitre plates which are commercially available. Due to higher than normal background reactions associated with special ELISA plates, they should not be used in this assay. 'U' bottom polystyrene plates are those of choice because of lower cost compared to flat-bottomed plates and capability of giving reproducible results with 50 uL reagent volumes.

4.2.2. Procedure

(a) Coating antigen dilutions are determined by checkerboard titrations (usually in the range 1:600 to 1:1000 depending on the batch).

(b) Dispense 50 µL of the antigen appropriately diluted in 0.1 mol/L sodium carbonate (pH 9.5) into each well of the plate, cover and allow to sit overnight at room temperature.

(c) Remove non-adsorbed antigen solution and wash the plates three times with PBS/Tween, preferably using either an automated plate washer or manually operated multichannel aspirator/dispenser. Alternatively, a plastic squeeze bottle can be used to direct a stream of diluent into each well, ensuring that complete flushing of each well is achieved. After the last washing the remaining fluid is removed from the wells by tapping the plate firmly upside down on a paper towel placed on the laboratory bench.

(d) Dilute test and control serum samples 1:200 by the addition of a minimum of 10 uL of serum to 2 mL PBS/Tween in glass test tubes. Thoroughly mix the tubes on a vortex mixer before adding 10 uL volumes to the microtitre plates. Use individual clean disposable plastic pipette tips for both the dilution and dispensing steps.
Forty serum samples are tested in duplicate on each plate in columns 1–10. The first serum sample is added to column 1 rows A/B, the 11th to column 1, rows C/D, etc. Columns 11 and 12 are reserved for the series of positive (rows C–H) and negative control serum dilutions (row B) and a reagent blank (row A) as indicated in Table 2. Positive control serum dilutions are made in test tubes prior to addition of 50 µL test volumes to each plate.

(e) Cover the plate(s) and incubate for one to two hours at room temperature.

(f) Wash plates(s) three times as above using PBS/Tween.

(g) Add 50 µL of conjugate diluted in PBS/Tween to all wells of the plates. The conjugate dilution to be used should be previously determined by checkerboard titration. A 1:200 dilution of the positive and

Table 2. Control sera dilutions and equivalent ELISA units

Row	*Serum dilution*	*ELISA units*
A	***	—
B	1:200**	0
C	1:51200*	4
D	1:25600*	8
E	1:12800*	16
F	1:3200*	64
G	1:800*	256
H	1:200*	1024

* Positive control.
** Negative control.
*** Reagent blank — no serum.

negative control sera should produce optical densities, in the system described, of between 1.5–2.0 and 0.1–0.2, respectively.

(h) Cover the plate(s) and incubate for one to two hours at room temperature.

(i) Wash plate(s) three times as above using PBS/Tween.

(j) Add 100 µL of substrate solution to each well, cover the plate(s) and incubate for about one hour at room temperature;

(k) Results can be assessed visually to within one dilution of the positive control serum or alternatively the optical density can be quantified using a commercially available microplate photometer. It may be necessary to mix the oxidised ABTS product by tapping a pencil against the side of the plate in those wells producing large colour changes. Substrate colour change is stable for up to several days as long as the plates are sealed with cellotape to stop evaporation.

4.2.3. Interpretation

The series of positive and negative controls are used to construct a standard curve relating optical density changes to ELISA units, 0–1024 (Table 2). Fig. 1 is a typical curve obtained. The optical densities of the test samples are then compared to those of these standards, either manually or by using a computer and a curve fitting program (e.g. Platereader Version 3.3, Regional Veterinary Laboratory, Benalla, Vic. 3672, Australia) to produce relative ELISA units. By definition, the relationship between respective serum sample ELISA units is linear, i.e. a sample of 100 ELISA units has an antibody 'titre' four times that of a sample of 25 ELISA units.

The lowest ELISA unit sample from an animal confirmed by bacteriological culture of semen to be infected with *B. ovis* is 20. However, ELISA unit values lower than this figure (down to 5) have been observed in experimentally infected animals about one week prior to confirmation of infection. Thus the following guidelines for interpretation of the test are given (Table 3).

Table 3. Interpretation of *Brucella ovis* ELISA titres

ELISA units	*Significance*
< 5	Negative
5-10	Doubtful
10-20	Suspect
> 20	Positive

4.2.4. Modifications

Suggested incubation times and volumes need not be rigidly adhered to. For example, sufficient antigen is bound to the plates after about one hour incubation and the conjugate incubation times may be decreased by altering the conjugate dilution or incubation temperatures.

5. Histopathology

Lesions in affected rams and foetuses are not pathognomonic but histopathological examination of tissue should form part of the evaluations of all cases presented for necropsy. Fixation of tissues in buffered formalin is satisfactory, however, preservation of the testis is much improved if Bouin's fixative (e.g. Lillie and Fullmer, 1976) is used.

In the ram, the epididymitis is usually confined to the cauda epididymidis and is characterised by predominantly mononuclear inflammatory cell infiltration of the interstitium with hyperplasia and vacuolation of the epididymal duct epithelium. Extravasation of spermatozoa with resultant spermatic granuloma formation and fibrosis is common in chronic cases. In the seminal glands, there is diffuse or focal mononuclear inflammatory cell infiltration.

Lesions in foetuses and placentas are also non-specific. There is an acute placentitis with necrosis of the placentome and focal areas of lymphoid cell hyperplasia in the foetal lung, liver, spleen and kidneys.

6. References

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