AUSTRALIAN STANDARD DIAGNOSTIC TECHNIQUES FOR ANIMAL DISEASES

STANDING COMMITTEE ON AGRICULTURE AND RESOURCE MANAGEMENT

ANIMAL HEALTH COMMITTEE

SUB-COMMITTEE ON ANIMAL HEALTH LABORATORY STANDARDS

Bovine Brucellosis

Serology

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The standard diagnostic techniques for the serological diagnosis of bovine brucellosis were previously published under five separate titles.

Complement Fixation Test for Bovine Brucellosis, by Working Party of Laboratory Officers, first published by the Australian Bureau of Animal Health (1975).

Reprinted by CSIRO for the Australian Agricultural Council (1987) as: Australian Standard Diagnostic Techniques for Animal Diseases, No. 2, *CFT – Bovine Brucellosis*.

Milk Ring Test for Bovine Brucellosis, by Working Party of Laboratory Officers, first published by the Australian Bureau of Animal Health (1975, revised in 1978).

Reprinted by CSIRO for the Australian Agricultural Council (1987) as: Australian Standard Diagnostic Techniques for Animal Diseases, No. 1, *Milk Ring Test – Bovine Brucellosis*.

Rose Bengal Test for Bovine Brucellosis, by Working Party of Laboratory Officers, first published by the Australian Bureau of Animal Health (1975).

Reprinted by CSIRO for the Australian Agricultural Council (1987) as: Australian Standard Diagnostic Techniques for Animal Diseases, No. 5, *Rose Bengal Test – Bovine Brucellosis*.

Brucellosis Serum Agglutination Test, by Working Party of Principal Laboratory Officers, first published by the Australian Bureau of Animal Health (1978).

Reprinted by CSIRO for the Australian Agricultural Council (1987) as: Australian Standard Diagnostic Techniques for Animal Diseases, No. 3, *Brucellosis – Serum Agglutination Test*.

Standard ELISA Test for Bovine Brucellosis, by P. Plackett and J. Stewart and first published by CSIRO for the Australian Agricultural Council (1986) as: Australian Standard Diagnostic Techniques for Animal Diseases, No. 6, *Standard ELISA test for Bovine Brucellosis*.

1. Introduction

Australia commenced the national campaign to eradicate bovine brucellosis in 1970, although control measures had been in operation already for several decades. Australia was declared free of bovine brucellosis in 1989. The control and eradication programs relied heavily on the detection of infected cattle by the identification of brucella-specific antibodies in serum.

Brucella abortus is the causative agent of bovine brucellosis. Contagious abortion, as it is commonly known, has a worldwide occurrence and was once one of the most serious diseases of dairy cattle in Australia where it caused considerable economic loss. This, together with the risk to the human population and the threat to our export trade in beef, were the prime motivations for the introduction of the national eradication campaign.

A definitive diagnosis of bovine brucellosis is achieved by the isolation of *B. abortus* from the foetus, uterine fluids or placenta from an aborting cow, or the uterus or udder and associated lymph nodes from an infected, non-pregnant cow. A presumptive diagnosis of bovine brucellosis is usually made by the application of one or more serological tests. The five serological tests used in the Australian eradication campaign are described below.

2. Brucella Milk Ring Test

2.1. General Information

The Brucella Milk Ring Test (BMRT) is very sensitive, detecting the presence of positive milk from an infected cow when it has been diluted with milk from non-infected animals. The test is, therefore, extremely valuable as a presumptive or screening test on bulk or can samples to locate potentially infected herds, thereby reducing the number of serological tests needed for accrediting herds. It is also useful, if carried out regularly, for the assessment of progress in any eradication or control program.

The development of a positive reaction depends on the following two processes.

(a) A 'fat globule agglutinin' normally present in milk aggregates the fat globules which then rise to the surface on standing. This is the normal cream layer.
(b) Stained brucella cells which are added as antigen are agglutinated if brucella antibodies are present in the milk. These aggregated stained cells adhere to the surfaces of the fat globules and rise with them to form a blue-coloured cream layer.

2.1.1. Brucella Milk Ring Test Antigen

The antigen is a haematoxylin stained suspension of killed *Brucella abortus* organisms. Phenol 0.5% w/v is added as preservative. The antigen is used to detect the presence of brucella antibodies in milk and cream. The antigen can be obtained from: FAO/WHO Collaborative Centre for Brucellosis, Central Veterinary Laboratory, New Haw, Weybridge, Surrey, UK).

2.1.2. Precautions in Testing Milk

The following precautions should be kept in mind when testing milk.

(a) Incorrect sampling could lead to excessive or insufficient cream content. This will interfere with the reading of the test.
(b) Excessive shaking denatures the 'fat globule agglutinin' and upsets the formation of the surface cream layer.
(c) Heating at 45°C or above reduces the brucella antibody content.
(d) Milk may be stored at 2–5°C for up to two weeks without undue loss of reactivity. Longer periods and/or higher storage temperatures cause antibody loss. Frozen samples may give false reactions.

2.1.3 False Positive Reactions

These may occur when the test is conducted on:

(a) freshly collected milk (the samples, therefore, must be refrigerated for at least 12 hours);
(b) milk from cows with mastitis;
(c) milk containing colostrum;
(d) milk from cows in the drying off period;
(e) milk from non-infected milking animals which have been vaccinated within the last three months with Strain 19 vaccine.

2.1.4. Preservative

Bulk milk samples are preserved with Bronopol (2-bromo-2-nitropropane-1,3-diol; marketed as 'Myacide' by Boots Co. and distributed in Australia by Harcos Chemicals Pty Ltd, 3 Alan Rd, Rydalmere, NSW 2116. Tel. (02) 684 4122).

Add 200 µL of a 5% solution of 2-bromo-2-nitropropane-1,3-diol in water (containing 0.188% carmoisine edicol or eosin yellow as an indicator) to 25–40 mL of milk or cream. Mix gently but thoroughly and hold the sample at 2–5°C.

2.1.5. Addition of Cream to Milk

Where the herd is composed of cows which produce milk of medium to high cream content, e.g. Jersey cows, it is not necessary to add extra cream to the test. Should the herd be one with a low fat production, e.g. Friesian, it may be necessary to add extra cream to the tests (0.1 mL of BMRT negative cream to each test).

2.1.5.1. Pooled negative cream

Obtain untreated milk from a brucellosis-free herd containing at least 25 lactating cows. Add 1 mL of formaldehyde (CH_2O) to each litre of milk, mix and allow the cream to rise by storing in a refrigerator overnight. Remove the cream.

Table 1. Number of cows in the herd to be tested

1–4 lactating:	Test the pool (1 mL test). If positive, test the milk from each cow after dilution 1:10 in negative BMRT pooled milk.
5–200:	Standard 1 mL test.
201–500:	Use 2 mL of milk in the test.
501–900:	Use 3 mL of milk in the test.
901 or more:	Arrange for breakdown of herd so that not more than 120 lactating cows are represented in one pool.

2.1.6. Herd Size

The volume of milk used in the BMRT varies with the size of the herd.

These figures (more than five cows; Table 1) refer to total herd size not to lactating animals. The optimum number of lactating animals represented when a 1 mL BMRT is done is 30–120 cows.

2.2. Test Procedure

2.2.1. Standard Brucella Milk Ring Test

A representative sample from each bulk tank or can is collected after ensuring that the contents are thoroughly mixed before sampling. The sample should be refrigerated at 2–5°C for at least 12 hours but preferably 48–72 hours before testing. Sour milk is unsuitable for testing.

On the day of test, remove the milk samples and the BMRT antigen from the refrigerator and hold at room temperature for one hour before testing.

Mix each sample gently but thoroughly to ensure even dispersion of the cream. For each sample, place 1.0 mL of milk in a tube having a diameter of about 10 mm (3/8 inch) to give a milk column of about 2 cm. Add one drop (30 µL) of the BMRT Antigen to each tube (hold delivery end of pipette vertically). Mix the contents of each tube gently but thoroughly within one minute of the antigen addition.

Place the tubes vertically in a rack in a constant temperature water bath at 37°C for one hour.

2.2.2. 2 mL and 3 mL Brucella Milk Ring Test

The procedure is as detailed above except that 2 or 3 mL of milk are used according to herd size. The antigen volume (30 µL) is unaltered. The incubation period is the same. Read and record the results as shown in Table 2.

2.3. Interpretation

Any degree of positive reaction is presumptive evidence of the presence of one or more infected animals in the herd. In a large herd, the presence of a very small number of infected animals may not be detected by the above technique unless their milk antibody levels are very high. It is recommended, therefore, that each herd be retested at intervals of one to three months. This procedure also includes cows which were not lactating at the time of previous testing.

2.4. Individual Animal Brucella Milk Ring Test

When positive BMRT results in the herd test are not confirmed by herd blood tests, it is useful to examine milk from individual animals in the herd. Any positive milk from an animal should be retested after diluting 1 in 10 in a pool of several negative milks (1 mL diluted milk + 30 µL antigen). A positive result in this test is indicative of infection.

2.5. Cream Brucella Milk Ring Test

Cream, as such, should not be tested by the standard BMRT. The following modification gives good sensitivity in detecting infected herds from which cream is produced. Sour cream is unsuitable for testing and results from cream that sours during testing should be disregarded.

The test is adversely affected by:

(a) pH below 5.0; and

(b) collection and storage periods which, when combined, exceed two weeks — this time is influenced by storage conditions and general hygiene.

2.5.1. Reagents

2.5.1.1. Precollection diluent

Saturated sodium bicarbonate solution,

NaH_2CO_3	20 mL
Formalin (37–40% CH_2O)	1 mL
Water	160 mL

Table 2. Interpretation of the Brucella Milk Ring Test

Cream ring	*Milk column*		*Reaction*	*Test result*
	No eosin	*Eosin in preservative*		
Intensively coloured blue	White	Pink	++++	Positive
Definitely coloured	Slightly coloured	Slight blue tone	+++	Positive
Definitely coloured	Moderately coloured	Moderate blue tone	++	Positive
Moderately coloured	Moderately coloured	Moderate blue tone	+	Positive
White or slightly coloured	Definitely coloured	Definite blue tone	–	Negative

2.5.1.2. Modified cream ring test neutraliser

(a) NaCl 8.5 g
H_2O 1 L
Dissolve the sodium chloride in the water and add sodium bicarbonate salt to give a saturated solution.

(b) Evaporated milk.

Mix equal parts of (a) and (b) as required. BMRT negative cream is pooled from at least 20 cows.

2.5.1.3. Brucella milk ring test antigen

2.5.2. Test Procedure

(a) Mix each cream sample gently but thoroughly.
(b) Place 0.6 mL precollection diluent (see 2.5.1.1.) in a centrifuge tube and add 4 mL cream. Mix.
(c) Refrigerate (2–5°C) until testing starts.
(d) At test time remove sample from refrigeration and warm to room temperature.
(e) Centrifuge each cream sample at about 1000 *g* for 15 min.
(f) Observe after centrifugation. Those which appear white (like skim milk) are classified as not sour. Those appearing colourless to slightly yellow are classified as sour and should be discarded.
(g) Remove 1.2 mL from the liquid layer. Avoid fat and milk solids as these interfere with the test. The liquid may be removed by using a needle or cannula, e.g. 15G x 4' and a 2 mL syringe.
(h) Add the 1.2 mL of liquid to 0.6 mL of the 'modified cream ring test neutraliser solution'(see 2.5.1.2.) in a 14 x 100 mm tube. Mix gently but thoroughly.
(i) Add 0.4 mL fresh BMRT negative raw cream. This cream should be a pool from at least 20 cows.
(j) Add 1 drop (30 µL) BMRT antigen and mix.
(k) Incubate in a waterbath for one hour at 37°C.
(l) Observe and record the results as for the BMRT.
(m) Interpret the test as for the milk BMRT.

3. Complement Fixation Test

3.1. Introduction

The technique of the Complement Fixation (CF) Test is modelled on that described in Anonymous (1965).

Titrations are carried out in tubes and the total volume is normally 1 mL (macrovolume). The diagnostic test is carried out in disposable plastic plates in volumes one-tenth of those used in the titrations (microvolumes). Various degrees of automation may be applied to this system. Warm fixation was chosen mainly for convenience. The 'prozone' problems that occur in this test need to be taken into account.

The system is extremely sensitive; the complement titration is capable of detecting much smaller differences in complement activity than are methods using 100% haemolysis as end point. To avoid differences in sensitivity between tests, great care is necessary in handling the various components, especially complement and erythrocyte suspension, and in the preparation of glassware used for titrations. When this care is taken, excellent reproducibility of results may be expected in the diagnostic tests. The titrations may at first sight appear complicated, but after a little practice they are rapidly and easily performed.

All glassware must be chemically clean, and preferably sterile. After use, glassware should be cleaned in detergent solution to remove all reagents and then placed in dilute chromic acid cleaning fluid for 18 hours [dilute chromic acid cleaning fluid comprises potassium dichromate ($K_2Cr_2O_7$), 20 g; sulfuric acid (H_2SO_4), 76 mL; distilled water to 1 L] or in a commercial preparation (such as RBS 25 or Decon) designed to serve the same purpose. It should then be rinsed thoroughly in tap water before finally being rinsed twice in distilled water. Glassware should be dried in the hot air oven.

3.2 Materials

3.2.1. Diluent

Barbital buffered salt solution is used in preparing all solutions and suspensions in the standardised CF test. The following method of preparation is taken from Kabat and Mayer (1961).

(a) Prepare a stock solution containing 1 mol/L magnesium chloride ($MgCl_2.6H_2O$) and 0.3 mol/L calcium chloride ($CaCl_2.2H_2O$), e.g. 20.33 g of magnesium chloride hexahydrate and 4.41 g of calcium chloride dihydrate made up to 100 mL in distilled water. Filter sterilise and store in the refrigerator in small amounts.
(b) Dissolve 85 g of sodium chloride and 3.75 g of sodium 5.5-diethyl barbiturate (barbital sodium, $C_8H_{11}N_2O_3Na$) in about 1.4 L of distilled water.
(c) Dissolve 5.75 g of 5.5-diethyl barbituric acid (barbital, $C_8H_{12}N_2O_3$) in about 500 mL of hot distilled water.
(d) Mix the solutions prepared in (b) and (c), cool to room temperature, add 5 mL of the magnesium and calcium stock solution described in (a), add distilled water to make a final volume of 2 L. This is the concentrated barbital buffer solution, which should be stored in a refrigerator.
(e) For use, one part of the concentrated buffer solution is mixed with four parts of cold distilled water. It is kept in the refrigerator until required. Freshly diluted buffer should be prepared each day. The pH of the diluted buffer should be 7.3–7.4. Some workers prefer to dilute the concentrated

buffer in four parts of sterile 0.04% gelatin solution rather than in distilled water. Tablets may be used for preparing barbital buffered salt solution.

Kolmer diluent, the formula for which is given in 8.1. may be used as an alternative.

3.2.2. Collection and Storage of Sheep Blood

Sheep known to produce erythrocytes of a consistently satisfactory level of sensitivity should be chosen and used exclusively. Blood is withdrawn under aseptic conditions into an equal volume of Alsever's Solution (see 8.2.) and thoroughly mixed. The sheep blood so preserved is stored aseptically in screw-capped bottles in the refrigerator and should not be used until at least five days after collection; thereafter, it may be used for up to one month. Sheep red blood cells in Alsever's solution are available commercially from: Filtrona Pty Ltd, PO Box 425, Altona North, Vic. 3205, Australia.

3.2.3. Washing the Erythrocytes

Up to 10 mL of the sheep blood stored in Alsever's solution is placed in a 50 mL centrifuge tube which is filled with diluent and the contents thoroughly mixed. The suspension is centrifuged to sediment the erythrocytes and the supernatant discarded along with the buffy coat, i.e., the thin layer of white cells that overlays the deposit. The erythrocytes are suspended in fresh diluent and the centrifugation repeated. For the third and final centrifugation the erythrocytes are resuspended in about 15 mL of diluent and centrifuged in a graduated tube at about 1000 *g* for 10 min. The deposit is used to prepare the suspension.

3.2.4. Standardisation of the Erythrocyte Suspension

The standardised erythrocyte suspension used should contain 0.95 g haemoglobin per 100 mL as determined by the cyanmethaemoglobin method described in 8.3. which is equal to a suspension containing $6x10^8$ erythrocytes as determined in an electronic cell counter. Such a suspension is equal to a 3% suspension of erythrocytes obtained by centrifuging a suspension of sheep erythrocytes at exactly 1000 g for 10 min in a graduated centrifuge tube and resuspending the deposit in 32.3x its volume of diluent.

In routine work the erythrocyte suspension is adjusted by using a spectrophotometer as described below, so that when 1 mL of the erythrocyte suspension is haemolysed in 15 mL of distilled water it will produce the required optical density (OD) on the machine; this is known as the target OD. The target OD for the particular machine being used is determined as described in 8.3.

3.2.5. Routine Preparation of the Standardised Erythrocyte Suspension

The deposit after the third centrifugation (see 3.2.3.) is suspended in about 27x its volume of diluent to make an erythrocyte suspension somewhat denser than that required for the standardised suspension; after thorough mixing, 1 mL of this suspension is haemolysed by mixing with 15 mL of distilled water and its OD determined.

The amount of diluent that needs to be added to the denser suspension to produce a suspension of the required density is calculated according to the formula:

Amount of diluent to be added (mL)=
(OD of denser suspension – Target OD)/
Target OD x No. mL to be diluted

e.g. when the OD of the denser suspension = 0.61, the target OD = 0.5 and the No. of mL of the denser suspension is 25, then the No. of mL of diluent to add is:

[(0.61 – 0.5)/0.5 x 25] or
[(0.11/0.5) x 25] = 5.5 mL

Each final suspension must be checked before use by haemolysing a sample (1 mL in 15 mL of distilled water) and verifying that the resulting suspension gives the target OD.

As an alternative, the standardised erythrocyte suspension may be prepared by carrying out the third centrifugation (see 3.2.3.) at exactly 1000 *g* for 10 min then suspending the deposit in 32.3x its volume of diluent. In this case the resulting suspension should be checked regularly, at least once a month, by determining the concentration of haemoglobin per 100 mL using the cyanmethaemoglobin method.

The standardised erythrocyte suspension if stored at about 4°C may be used for up to 24 hours after preparation or for a longer period provided there is no evidence of lysis in the suspension.

3.2.6. Haemolysin (Amboceptor)

The haemolysin is titrated to determine the concentration which, when added to an equal volume of standardised erythrocyte suspension, will produce an optimally sensitised erythrocyte suspension.

The haemolysin should be prepared in rabbits. Details of the technique of producing haemolysin may be found in most text books giving serological techniques, e.g. Campbell *et al.* (1970) or Cruickshank *et al.* (1973). Haemolysin is also available commercially usually in liquid form preserved with an equal quantity of glycerine. (Haemolysin is available in Australia from: Edward Keller Aust., Pty Ltd, Medical Division, Private Bag 3, Mordialloc, Vic. 3195; and Gilles Plains Animal Resource Centre, 101 Blacks Rd, Gilles Plains, SA 5086.) Haemolysin preparations of less than a titre of 1:500 should not be used.

In use, a 1:100 stock dilution of glycerinated haemolysin may be made in CF diluent and stored in frozen aliquots. The haemolysin is titrated each time a batch of 1:100 dilution is made and each time a new batch of erythrocytes is brought into use.

3.2.7. Titration of the Haemolysin

(a) Prepare the standardised erythrocyte suspension as already described (see 3.2.2.–3.2.5.).

(b) From the 1:100 stock dilution of haemolysin prepare the following range of dilutions in CF diluent: 1 in 500, 1000, 1500, 2000, 3000, 5000 and 10 000. For example:
Add 1 mL 1:100 dilution of haemolysin to 4 mL diluent to give a 1:500 dilution
Add 1 mL 1:100 dilution of haemolysin to 9 mL diluent to give a 1:1000 dilution
Add 2 mL 1:1000 dilution of haemolysin to 1 mL diluent to give a 1:1500 dilution
Add 1 mL 1:1000 dilution of haemolysin to 1 mL diluent to give a 1:2000 dilution
Add 1 mL 1:1000 dilution of haemolysin to 2 mL diluent to give a 1:3000 dilution
Add 1 mL 1:1000 dilution of haemolysin to 4 mL diluent to give a 1:5000 dilution
Add 1 mL 1:1000 dilution of haemolysin to 9 mL diluent to give a 1:10 000 dilution.

(c) Add 1 mL of each haemolysin dilution to 1 mL of standardised erythrocyte suspension while gently agitating the erythrocyte suspension, then leave the mixtures at 37°C for 15 min to allow sensitisation of the erythrocytes to occur, agitating the tubes every five minutes.
The rest of the procedure is done in duplicate to minimise pipetting errors, the tubes being kept cold, while the reagents are being dispensed, e.g. stood in a polystyrene foam container containing crushed ice.

(d) To each of a duplicate series of seven tubes add 1.0 mL of diluent and 0.5 mL of complement diluted in such a way that it will produce about 70–80% haemolysis with the more concentrated haemolysin dilutions (with good quality complements a 1:350 dilution is satisfactory).

(e) From each of the haemolysin–erythrocyte mixtures in turn 0.5 mL is transferred to each of a pair of tubes containing complement and diluent and thoroughly mixed.

(f) Incubate the tubes for 30 min in a water bath at 37°C with gentle shaking after 15 min.

(g) Remove the tubes from the water bath, add 2 mL of cold diluent to each, then centrifuge the tubes to deposit any erythrocytes remaining unlysed.

(h) Pour off the supernatant from each tube and read the ODs in a spectrophotometer. This results in duplicate readings being obtained for each haemolysin dilution tested.

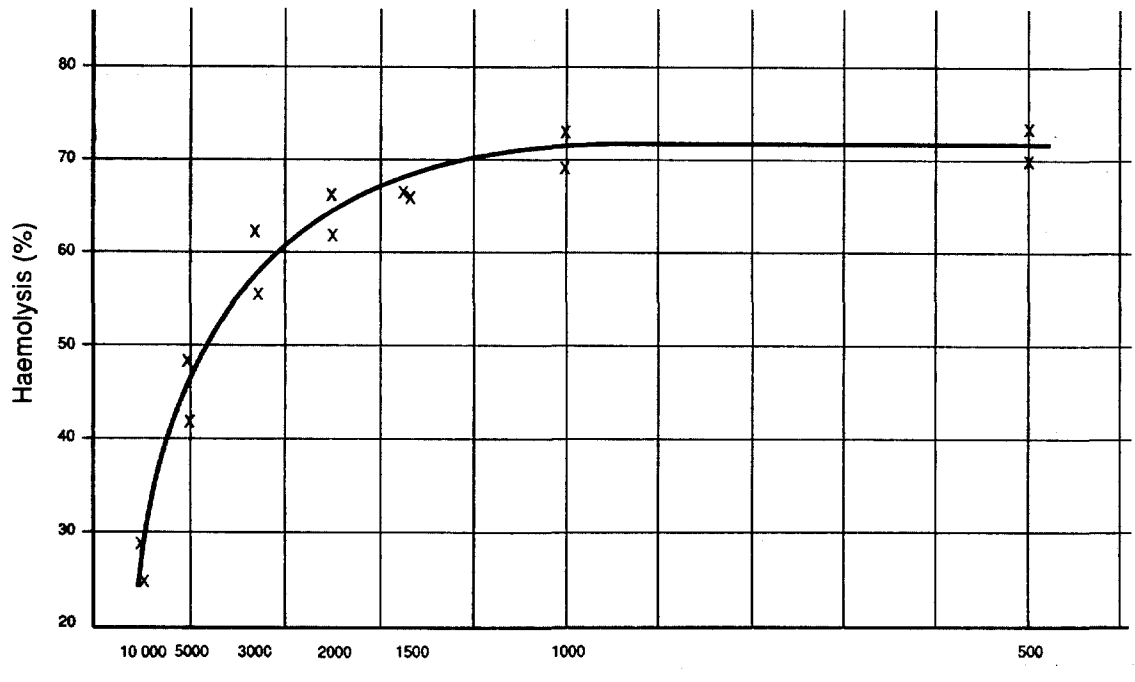

Figure 1. Titration of the haemolysin.

(i) The OD given by 100% haemolysis is the same as the target OD used in preparing the standardised erythrocyte suspension.

(j) Calculate the percentage of haemolysis for each tube in the titration, e.g. supposing the OD produced by 100% lysis is 0.5, a tube showing an OD of 0.21 would have 0.21x 100/0.5 = 42% haemolysis.

(k) Plot the percentage haemolysis given by each dilution on graph paper marked out as shown in Fig. 1. To calibrate the abscissa, measure an arbitrary distance, say 10 scale divisions, from the left hand extremity and place a point here representing the 500 dilution; this is the extreme right-hand end of the abscissa. Distances from the left-hand end for the points representing the other dilutions are calculated by dividing the reciprocal of the dilution in 500, e.g. the point for the 1000 dilution is placed 500/1000 or half way along the line, the 5000 dilution 500/5000 or one-tenth of the way along etc. The percentage haemolysis is marked linearly along the ordinate, the points for percentage haemolysis are plotted for each haemolysin dilution and the line drawn, ignoring outlying points.

(l) Optimal dilution of haemolysin for use in the test is decided by determining where the plateau begins (1:1000 in Fig. 1) and selecting a dilution about 25% more concentrated (1:800 in the example given) for use in the test. The selection of the quantity of haemolysin to use in the test is not critical so long as an ample amount is chosen. The quantity of haemolysin forms a fixed point against which the amount of complement to be used is determined accurately.

3.2.8. Complement

At least four guinea pigs should be bled, the serum separated as soon as practicable from the clot and pooled to produce complement. Adult guinea pigs receiving adequate green food produce good quality complement but all food should be withheld during the 12 hours preceding bleeding; pregnant females or those that have recently given birth should not be used.

Complement may be preserved by Richardson's method and when so preserved will maintain its titre for about six months if stored at 4°C. Even at room temperature the loss of titre is not rapid.

Two stock solutions which keep indefinitely are used:

(a) Solution A

Boric acid, H_3BO_3	0.93 g
Borax, $Na_2B_4O_7.10H_2O$	2.29 g
Sorbitol, $C_6H_{14}O_6.1/2\ H_2O$	11.74 g
Saturated NaCl solution to	100 mL

(b) Solution B

Borax	0.57 g
Sodium azide, NaN_3	0.81 g
Saturated NaCl solution to	100 mL

To preserve complement, mix eight parts of guinea pig serum with one part of (b), followed by 1 part of (a). Before use it is necessary to restore tonicity by adding one part of preserved complement to seven parts of distilled water. This gives a 1:10 dilution of complement.

Unpreserved complement may be stored frozen at -70°C or below, storage in liquid nitrogen is satisfactory, or in the dried state. Dried complement is also available commercially in Australia from: ICN Biomedicals, Unit 12/31 Sevenhills Road, North Sevenhills, NSW 2247; and Gilles Plains Animal Resource Centre, 101 Blacks Rd, Gilles Plains, SA 5086. Complement should be stored in the refrigerator or freezer.

3.2.9. Titration of Complement

Complement is titrated: (a) before using a new batch of complement; and (b) before using a new collection of sheep blood to prepare the erythrocyte suspension. The quantity of complement required to lyse 50% of optimally sensitised erythrocytes is determined, this is called 1 $C'H_{50}$; 5 $C'H_{50}$ are used in the test. A master dilution of complement is prepared. When complement preserved by Richardson's method is being used this will be the 1:10 dilution produced by reconstitution in distilled water. The master dilution should be made in fluid at refrigerator temperature (2–5°C) and stored at this temperature. A small quantity of the master dilution is further diluted to produce the titration dilution; with good quality complement the titration dilution is likely to be 1:350 but this may have to be determined by trial.

The procedure for the titration is as follows:

(a) Prepare sensitised erythrocytes by mixing equal volumes of standardised erythrocyte suspension and haemolysin diluted as determined in 3.2.7. Allow the mixture to stand at 37°C for 15 min, mixing every five minutes.

Table 3. Complement titration — arrangement and quantities for tube titration

	Tubes					
	1	*2*	*3*	*4*	*5*	*6*
Complement 1 in 350 mL	0.3	0.4	0.5	0.6	0.7	0.8
Diluent mL	1.2	1.1	1.0	0.9	0.8	0.7
Place in water bath at 37°C for 30 min then add						
Sensitised erythrocytes mL	0.5	0.5	0.5	0.5	0.5	0.5

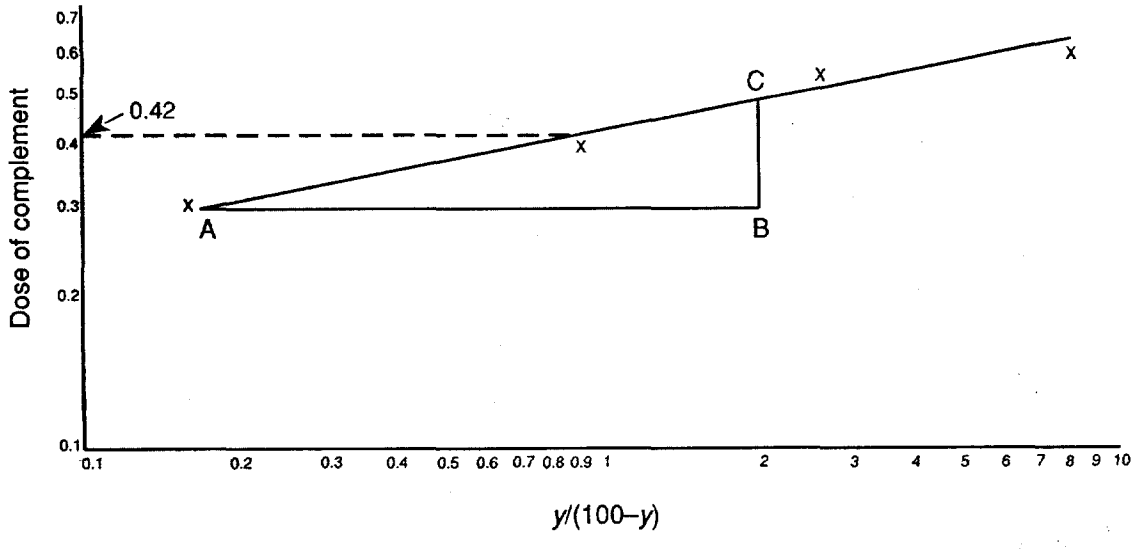

Figure 2. Calculation of the dose of complement to produce 50% haemolysis.

(b) Prepare master and titration dilutions of complement.

(c) This procedure is done in duplicate to minimise pipetting errors. The test is done in twice the standard volume to minimise errors in pipetting and to produce a total volume large enough to give a reading on the spectrophotometer. Arrange six suitable tubes in a rack and add the reagents in the quantities and the order shown in Table 3. After the diluent has been added the contents are mixed thoroughly and the tubes are placed in a water bath at 37°C for 30 min.

(d) After the addition of the sensitised erythrocytes the contents of each tube are mixed by gentle agitation then incubated in a water bath at 37°C for 30 min, during which time they are agitated at least once. After the incubation is completed the tubes are removed from the water bath, 2.0 mL of cold diluent is added to each tube and the tubes centrifuged to deposit the unlysed cells. The supernatants are then poured off and their ODs determined in the spectrophotometer.

Supposing the OD corresponding to 100% lysis is 0.5, a reading of 0.2 would indicate:

$$[(0.2 \times 100)/0.5] = 40\% \text{ haemolysis}$$

Table 4. Calculation of the percentage of haemolysis in complement titration

Dose of complement	*Optical density (OD)*	*Percentage haemolysis (y)*	*y /(100-y)*
0.3	0.06	13	13/87= 0.15
0.4	0.22	47	47/53 = 0.89
0.5	0.34	72	72/28 = 2.6
0.6	0.42	89	89/11 = 8.1

(e) The dose of complement required to produce 50% haemolysis may now be determined graphically by plotting the degree of haemolysis for each dose of complement used in the titration on log/log paper, i.e. paper with log scales both vertically and horizontally (Fig. 2). The degree of haemolysis is calculated for each tube from the expression $[y/(100\text{-}y)]$ where y is the percentage haemolysis (only tubes showing between 10 and 90% haemolysis are taken into consideration).

(f) The values for the expression $[y/(100\text{-}y)]$ are plotted for each dose of complement (Fig. 2) and a straight line fitted through the points. The point where the line 'AC' has a value of 1 on the abscissa indicates the 50% haemolytic dose ($C'H_{50}$) and this may be read from the ordinate (broken line in Fig. 2). The calculation is made as in the example given in Table 4.

(g) A simple calculation suffices to arrive at the dilution factor required for the diagnostic test. In our example 0.42 mL of a 1:350 dilution contains 1 $C'H_{50}$; therefore 5x 0.42, i.e. 2.1 mL of this dilution will contain five $C'H_{50}$ and to calculate the dilution that will contain this amount of complement in 0.5 mL (here we are using double volumes) the following equation is used wherein x is the dilution factor required:

$$350/2.1 = x/0.5; \text{ i.e. } x = 83.$$

This formula may be simplified to:
x (dilution factor) =[titration dilution of complement/10x50% haemolytic dose]
i.e. in our example: 350/4.2 = 83
Smaller volumes being used in the diagnostic test does not affect this dilution factor since

the proportions of each reagent remain the same. The 1/10 dilution of complement now being stored in the refrigerator needs, therefore, to be diluted 83/10, i.e. 8.3x, to bring it to the 1:83 required for use in the diagnostic test, i.e. each 1 mL of the 1:10 dilution is added to 7.3 mL of diluent. The 1:10 dilution will maintain its potency throughout the working day if stored as described.

(h) To save repeated calculations a table of the percentage haemolysis and $[(y/100)\text{-}y]$ for each spectrophotometer reading should be drawn up (see 8.4.).

(i) The validity of the slope of the line is now examined. A horizontal line 10 cm long (line AB in Fig. 2) is drawn with its left hand extremity touching the slope at a convenient point. From the right hand end of this horizontal line at B a vertical line is extended up to the point C where it meets the slope. With a satisfactory slope the length of the vertical line should be one-fifth of the length of the horizontal line ± 10%, i.e. not more than 22 mm and not less than 18 mm long. Though complement giving slopes steeper or flatter may give satisfactory results in the diagnostic test, experience has shown that accurate and reproducible results are more likely to be obtained if only complements satisfying these criteria are used.

3.2.10. Antigen

The antigen for the CF test is available from: FAO/WHO Collaborative Centre for Brucellosis, Central Veterinary Laboratory, New Haw, Weybridge, Surrey, UK. The antigen should be diluted freshly for each day's test.

3.2.11. Inactivation of Sera

In addition to the natural anticomplementary activity present in serum, certain conditions, particularly bacterial contamination, may produce an added degree of anticomplementary activity. Haemolysis in serum has little, if any anticomplementary effect. The anticomplimentary activity due to bacteria may be greatly reduced by centrifuging the serum at 8000 *g* or greater for 15 min (or equivalent). Other methods of removing anticomplementary activity are available.

The natural anticomplementary activity in bovine serum is removed by heating at 58°C for 30 min in a water bath. Serum may also be inactivated in the trays (see 3.3.) in an incubator at 58°C. If this is to be successful it is necessary to ensure that: (a) the plates are not stacked more than three high; (b) the incubator door is not opened during inactivation; and (c) inactivation begins when the temperature of the incubator regains 58°C — a fan installed in the incubator aids the recovery of the operating temperature after the door has been opened.

3.3. Test Procedure

The test is carried out in disposable plastic microtitre plates with U-shaped wells. The volumes of reagents used are one-tenth of those used in the titrations. Owing to the frequent occurrence of 'prozones', serum dilutions up to at least 1:128 should be tested. Normally 12 sera are tested on each plate; this provides eight wells per serum.

For automated systems the following procedure is recommended. A convenient amount of undiluted serum (at least 50 µL) is placed in the first well of the row, the wells containing serum are covered with tape and inactivated in an incubator at 58°C for 30 min. After inactivation, 25 µL of diluent is added to each well except the first of each row, doubling dilutions are made mechanically, starting with the undiluted serum and continuing right across the plate. Antigen (25 µL) is added to each well from the third to the end of each row. The second well acts as an anticomplementary control and requires the addition of a further 25 µL of diluent to compensate for lack of antigen. Complement (25 µL) is added to each well except the first of each row. (The test is read from the 1:4 dilution, i.e. the third well, onwards; that the anticomplementary control is more concentrated than the first dilution of the test proper is not considered to have practical significance. Sera judged as anticomplementary in this system can be retested manually).

Antigen and complement may be mixed and added together. The reagents are mixed by tapping the plate gently, the plates are covered, e.g. by another plate, and placed in an incubator at 37°C for 30 min. The sensitised erythrocyte suspension is prepared by mixing equal volumes of standardised erythrocyte suspension and haemolysin dilution and keeping the mixture at 37°C for 15 min, agitating gently every three to five minutes. After the plates are removed from the incubator 25 µL of sensitised erythrocyte suspension is added to each well except the first of each row. It is important that the sensitised erythrocytes are thoroughly mixed with the other components and kept in suspension; this can be best achieved by using a mechanical shaker installed in the 37°C incubator. After shaking for a further 30 min at 37°C the plates are removed from the incubator. They are either centrifuged at 2000 *g* for 10 min in the special microtitre carrier plates or placed in the refrigerator for at least two to three hours to ensure deposition of any unlysed erythrocytes. The results can now be read; this may be done over a white diffused light or from below using a magnifying mirror.

3.4. Interpretation

When the reaction in any well is negative, the contents are completely clear and pink due to haemoglobin released from the lysed cells. Complete fixation in the absence of haemaggluti-

Table 5. Standards for agglutination tests

	Degree of agglutination				
	++++	+++	++	+	–
Phenol-saline (mL)	1	0.75	0.5	0.25	0
Antigen, diluted 1:2 (mL)	0	0.25	0.5	0.75	1.0
Simulated percentage clearing	100	75	50	25	0

nation appears as a compact button of erythrocytes in the centre of the well surrounded by clear, colourless fluid; lesser degrees of fixation produce smaller buttons of erythrocytes and pink fluid. When agglutination of unlysed erythrocytes occurs, and this is uncommon beyond the 1:8 dilution, there is a film of erythrocytes covering the whole of the bottom of the well. This may be a very fine film when the degree of fixation is only slight; these are nevertheless regarded as positive reactions of varying degrees.

A control serum of moderate titre (say 1:16) is tested in each row of a plate. Such a control plate is included with each set of tests or after every 20 plates.

A complement control may be set up. Working dilution (50 µL) of complement and 0.7 mL of diluent are placed in each of four tubes. These are incubated for 30 min in the water bath at 37°C, after which 0.25 mL of sensitised erythrocytes are added. After a further 30 min in the water bath, 1 mL of cold diluent is added, the tubes centrifuged and the average percentage haemolysis of the supernatants determined. This average should represent between 25 and 75% haemolysis.

4. Serum Agglutination Test

4.1. Introduction

The serum agglutination test (SAT) used in Australia is the European Tube Agglutination Test described by Alton *et al.* (1988). The technique for carrying out the test is described in 4.2. Details of the production and standardisation of the antigen may be found in the original publication.

4.2. Test Procedure

The tests may be done in either glass or plastic tubes suitable for working with 1 mL volumes. In view of the occasional occurrence of prozone phenomena, at least five tubes are normally used for each serum under test. Using an automatic pipette for preference, 0.8 mL of phenol–saline (0.85% sodium chloride and 0.5% phenol) is placed in the first tube and 0.5 mL in each succeeding tube; 0.2 mL of the serum under test is transferred to the first tube and mixed thoroughly with the phenol–saline already there; 0.5 mL of the mixture is carried over to the second tube from which, after mixing 0.5 mL is transferred to the third tube, and so on. This process is continued until the last tube, from which, after mixing, 0.5 mL of the serum dilution is discarded. This process of doubling dilutions results in 0.5 mL of dilutions 1:5, 1:10, 1:20 and so on, in each tube. To each tube is then added 0.5 mL of antigen at the recommended dilution and the contents of the tube are thoroughly mixed, thus giving final serum dilutions of 1:10, 1:20, etc. The tubes are then incubated at 37°C for 20 ± 1 hour before the results are read. The dispensing, mixing, and transferring of the serum under test may be done with a pipette, but these operations are more conveniently carried out with a 1 mL tuberculin syringe fitted with a needle that has had its beveled tip removed so that the tip just fails to reach the bottom of the tube. The hypodermic needle may be replaced by fine polythene tubing (Intramedic PE 50) fitted into the nozzle of the syringe and cemented in place; the 'dead space' with this type of tubing is negligible.

The degree of agglutination is assessed on the amount of clearing that has taken place in the tube as compared with a standard tube. The tubes are examined without being shaken against a black background with a source of light coming from above and behind the tubes. Complete agglutination and sedimentation with water-clear supernatant is recorded as ++++, nearly complete agglutination and 75% clearing as +++, marked agglutination and 50% clearing as ++, some sedimentation and 25% clearing as +, and no clearing as –.

The accuracy and reliability of the readings are much improved if standard tubes simulating degrees of agglutination are available for comparison. Standards should be prepared at the time the tests are done and incubated with them. The antigen is diluted by mixing 2 mL of antigen, diluted as for the test, with 2 mL of phenol–saline; the five standard tubes are prepared by mixing the quantities shown in Table 5.

4.3. Interpretation

The results of the agglutination tests should be expressed in international units (IU) (see 4.4.), and interpreted according to the recommendations contained in the fifth report of the FAO/WHO Expert Committee of Brucellosis (FAO/WHO, 1971), which recommends that in cattle the minimum diagnostic level be 100 IU/mL for cattle ≤ 8 months of age and not vaccinated with strain 19. Levels 50% lower than these, i.e., 50 IU/mL for non-vaccinated and 100 IU/mL for vaccinated animals, should be regarded as 'doubtful' or 'suspicious'. Such animals should be retested after 60 days. Supplementary diagnostic tests are often helpful in deciding the status of animals that are classified as doubtful by agglutination tests.

4.4. The International Unitage System

By definition the International Standard anti-*Brucella abortus* serum (ISAbS) contains 1000 IU per ampoule. Therefore, using an antigen that gives a titre of 1:500 with the ISAbS, a serum under test giving a titre of 1:40 contains 1000x 40/500 = 80 IU/mL. Table 6 shows the conversion of titres to IU/mL.

5. Rose Bengal Test

5.1. Introduction

The Rose Bengal test (RBT) is a spot agglutination test used to screen herds. For the diagnosis of individual cattle the test is over sensitive, especially in cattle vaccinated with strain 19.

5.2. Materials

The test should be done on clear glass or plastic plates, marked with 15 mm squares delineated by araldite, or in WHO haemagglutination trays or equivalent.

All glass plates or plastic trays should be perfectly clean. After use, rinse immediately under the tap to remove all obvious residues. Soak in suitable detergent solution (RBS 25 or Decon) for at least two hours (preferably overnight). Rinse thoroughly in tap water and finally rinse twice in distilled water. Then dry in incubator or drying chamber (such as commercial clothes dryer).

The RBT antigen is obtained from the Central Veterinary Laboratory, Weybridge (see 3.2.10.).

5.3. Test Procedure

Serum samples and RBT antigen should be at room temperature. Because not all laboratories are air conditioned, no upper limit can be set for conducting the test, but around 20°C is desirable. The temperature should not be below 15°C.

Dispense serum samples in drops with an Eppendorf type pipette with a disposable tip. The volume dispensed to be 30 µL for the flat glass or plastic plates and 25 µL for the WHO haemagglutination trays.

Use the antigen at the concentration recommended by the manufacturers. Dispense the antigen with a dropping pipette. The volume must be equal to that of the serum sample.

To minimise delay between the adding of antigen to the first serum sample and the last serum sample, no more than one plate or tray per operator should be set up at the one time. The commencement time should be marked on the plate.

Immediately after the addition of the last drop of antigen to a plate or tray, each test should be mixed thoroughly by the method found most convenient in the testing laboratory.

With WHO trays, the following procedure is satisfactory:

Place the tray on a flat surface and hold squarely with both hands. Mixing of the tests is achieved by moving the hands first in a clockwise direction and then anticlockwise with increasing vigour, taking care to avoid spillage. This results in a swirling effect and the test mixture must cover the whole of the bottom of the well. With flat trays, mixing may be by gentle rocking and tapping of the trays, or by stirring with clean toothpicks. Mixing should be carried out over a template so that an adequate *uniform* area is covered by the test mixture. After mixing, the plate or tray is immediately placed on a rocker.

The test plate should be rocked mechanically for four minutes at a rate of about 30 oscillations per minute. The tests should then be read without delay. When reading the tests the plate should be slowly tilted back and forth over a light source of even intensity which may be indirect. A suitable system of double checking the readings should be used to reduce or eliminate operator error.

5.4. Interpretation

A scoring system for results should be used, for correlation with CF test results. The following allows distinction of degree of reaction:

0 — No agglutination, no rimming, uniform pink colour;

1 — Barely perceptible agglutination and/or some rimming;

2 — Fine agglutination, definite rimming, Some clearing;

3 — Coarse clumping, definite clearing.

Samples giving reactions of 1, 2 or 3 should be subjected to the CF test.

Table 6. Conversion of titres to International Units/mL for an antigen giving a titre of 1:500 with the International Standard Anti-*Brucella abortus* Serum (ISAbS)

Final dilution of serum	*End point reading*	*IU/mL*
1/10	1+	17
	2+	20
	3+	23
	4+	27
1/20	1+	34
	2+	40
	3+	47
	4+	53
1/40	1+	67
	2+	80
	3+	93
	4+	106
1/80	1+	134
	2+	160
	3+	186
	4+	212
1/160	1+	268
	2+	320
	3+	372
	4+	424
1/320	1+	536
	2+	640
	3+	744
	4+	848
1/640	1+	1072
	2+	1280
	3+	1488
	4+	1696

5.5. Standardisation

The sensitivity of the test should be checked each day by putting up a standard working positive serum. The serum is available from: FAO/WHO Collaborative Centre for Brucellosis, Central Veterinary Laboratory, New Haw, Weybridge, Surrey, UK.

This serum should give a '3' reaction when undiluted and a '2' and '1' reaction at nominated dilutions. If the serum does not give these reactions, testing should not be proceeded with, and the cause of the incorrect reactions should be investigated.

The sensitivity of the batch of antigen can be checked against a battery of sera giving different degrees of reaction. The pH of the antigen should be 3.65 ± 0.095. The buffering capacity of the antigen can be checked by mixing one volume of antigen with one volume of freshly collected bovine serum. The pH should remain below 4.0.

6. Enzyme-Linked Immunosorbent Assay

6.1. Introduction

The enzyme-linked immunosorbent assay (ELISA) for bovine brucellosis is a simple and sensitive assay for diagnostic use. Significant titres usually develop soon after initial infection and persist for the life of the animal.

Large changes in the relative proportions of the different isotypes occur during the course of a response but do not impinge on the performance or the interpretation of the assay. The optimum cut-off point for diagnosis of infection in unvaccinated cattle is about 64 N units/mL (i.e. a 32 000-fold dilution of the NSW standard serum) with commercial antibovine IgG (H + L chain) reagents.

6.2. Reagents

6.2.1. Phosphate Buffered Saline, pH 7.2

Phosphate buffered saline (PBS) is made up by mixing:

Sodium dihydrogen phosphate, $NaH_2PO_4.2H_2O$ 28 mL
0.2 mol/L (31.8 g/L)

Disodium hydrogen phosphate, $Na_2HPO_4.7H_2O$ 72 mL
2 mol/L (53.6 g/L)

and adding a solution of 8.0 g of sodium chloride in 900 mL of glass-distilled water.

The heptahydrate is specified because of its stability in air. The anhydrous salt is hygroscopic and will absorb two to seven moles of water per mole depending on temperature and humidity. The dodecahydrate readily loses five moles of water.

6.2.2. PBS + Tween 20 (PBST)

PBST is made up from PBS with the addition of 0.5 mL of Tween 20 per litre.

6.2.3. Antigen

The crude antigen, consisting mainly of lipopolysaccharide (LPS), is prepared from smooth *Brucella abortus* (strain 19) cells by the method of Berman *et al.* (1980). ELISA LPS *Brucella abortus* antigen is available from: Dr J. E. Searson, Regional Veterinary Laboratory, Department of Agriculture, Wagga Wagga, NSW, 2650, Australia. The product requires treatment with alkali before use. The extraction procedure and the method of alkali treatment are described in 8.5.

6.2.4. Enzyme-labelled Antiglobulin Conjugates

Suitable preparations of antibovine IgG (H + L chains) or F(ab)2, conjugated to horseradish peroxidase (HRP) or to alkaline phosphatase (AP), are obtainable from commercial sources. Conjugates are stored at -10 to -15°C, after addition of an equal volume of glycerol to prevent freezing.

The optimum working dilution of each batch of conjugate is determined by checker-board titration against serial dilutions of the reference serum on an antigen-coated tray.

6.2.5. Substrates

5-Aminosalicylic acid (5-AS, MW 153), o-phenylenediamine (oPD, MW 108), and 2,2'-[azinodi(3-ethylbenzthiaxoline sulfonate)] (ABTS) can all be used with HRP-conjugates. 5-AS can be conveniently purified and made up as a stock solution at 1 mg/mL and pH 6, by the method of Ellens and Gielkens (1980). It is less toxic than oPD (Sax, 1984) and is less likely to cause skin sensitivity. The stock solution, without added hydrogen peroxide, can be stored frozen in convenient amounts and thawed as required.

A 0.1 mol/L solution of hydrogen peroxide is prepared shortly before use by diluting 0.5 mL of 30% (8.8 mol/L) H_2O_2 solution (analytical reagent, AR grade) with 43.5 mL of distilled water. The concentration of peroxide solution is easily checked by measuring the absorbance at 240 nm, at which the molar absorbance is 43.6. Just before use, 0.2 mL of the 0.1 mol/L solution is added for each 10 mL of the stock 5-AS solution.

The preferred substrate for AP-conjugates is p-nitrophenyl phosphate, dissolved in 1 mol/L diethanolamine buffer pH 9.8, to a concentration of 1 mg/mL.

6.2.6. Stopping Reagents

Under the usual assay conditions, the peroxidase-catalysed reaction is roughly linear for only a short period at room temperature. The decline in rate is faster if the peroxide concentration or the temperature is raised. Except for very short incubation times, the decline more than offsets any increase in the initial rate due to changes in these variables. After incubation for 30 min the

rate of reaction is low and the trays may be read without the addition of stopping reagents. The 5-AS reaction product is read at 450 nm.

Reactions catalysed by AP can be stopped by adding sodium hydroxide before reading the absorbency at 405 nm.

6.2.7. Reference Serum

The contents of an ampoule of freeze-dried NSW standard anti-*B. abortus* reference serum (batch Nos. 20083/20085) are reconstituted in 1.0 mL of pure water. (Batch Nos. 20083 and 20085 are available from: Dr J.E. Searson, Regional Veterinary Laboratory, Department of Agriculture, Wagga Wagga, NSW 2650, Australia.) This is then diluted 200-fold in PBST and 0.5 mL volumes are dispensed into screw cap plastic vials and stored at -20°C. For use, the contents of a vial are thawed and diluted to 5.0 mL in PBST. This solution, which is a 2000-fold dilution of the reconstituted serum, is assigned an antibody concentration of 1024 'N units'/mL.

6.2.8. Microtitre Trays

Some brands, including some marketed specially for ELISA work, show high levels of non-specific binding. Batch to batch, tray to tray and well to well variation are common. Several samples from any source should be tested before a particular batch is accepted for routine use. Brands found acceptable include the flat-bottomed Disposable Products ELISA tray and Linbro-Titertek catalogue No. 76-301-05 [available in Australia from Disposable Products, 810 Princes Hwy, Springvale, Vic. 3171. Tel. (03) 548 4411]. Some brands of trays with round-bottom wells may also be usable, in which case reagent volumes per well may be reduced.

6.3. Method

(a) A solution containing 1 µg/mL of crude alkali-treated *B. abortus* LPS in PBS is added to the wells (100 µL for flat-bottomed wells and 50 µL/well for round-bottomed wells). Trays are covered with cellotape or with lids and shaken at 37°C for one hour, or kept overnight, with or without shaking, at 4°C.

(b) Antigen solution is shaken out and the trays are washed four times at room temperature. If automatic washing machines are not available, each wash is done as follows. The wells are filled with PBST and left for about one minute on the bench. Wash fluid is then shaken out and the operation is repeated. After the fourth wash, the trays are placed face down on clean filter paper and allowed to drain for 5–10 min.

(c) Serum dilution in PBST is placed in each of two wells (100 µL for flat-bottomed wells and 50 µL/well for round-bottomed wells). Unknown sera are diluted 200-fold and placed in adjacent wells. e.g. in rows A and B, starting at the top of column 2 of the tray. Column 1 is left empty until substrate is added before the final incubation step. It is used to set the blank for each optical channel before reading on the microtitre reader. The placing of replicate serum samples in adjacent optical channels minimises the effects of any errors in the blanks and makes such errors easily detectable by inspection of the readings. It also helps to detect edge-effects (i.e. anomalous behaviour of samples in the wells at the edges of the trays). Duplicate samples of seven serial two-fold dilutions of standard reference serum, or of a working reference serum calibrated against the standard, (starting with a 2000-fold dilution of the neat NSW reference serum) and of diluent alone, are placed in columns 6 and 7 of the tray. Further unknown samples are placed in columns 8–11, and column 12 receives PBST only. If the test is to be read by eye and the only information sought is whether or not the test sera exceed a predetermined level of reaction, then the inclusion of standards diluted to that level are all that is necessary. If the trays are to be read by spectrophotometer and a curve fitting program is being used to give the maximum precision, then standards at six or more different levels may be needed.

(d) Trays are covered and kept at room temperature for 1.5 hours. If a more rapid test is needed, can incubate for 45 min at 37°C in a fan-forced incubator with constant mechanical shaking of the trays. An incubator with fan-driven air circulation is advisable to ensure a constant and uniform temperature for all trays. Trays should not be stacked more than two high during incubation.

(e) Serum is shaken out and the trays are drained and washed four times as in (b).

(f) A suitable dilution of enzyme-antiglobulin conjugate in PBST is added to each well (100 µL for flat-bottomed wells and 50 µL/well for round-bottomed wells). Trays are covered and incubated at room temperature for 1.5 hours.

(g) Conjugate is shaken out and trays are washed four times and drained as in (b).

(h) Substrate solution (100 µL) is added to each well and trays are covered and shaken at room temperature for 30–60 min depending on the level of sensitivity required. The sensitivity of the test should be such that the cut-off point for diagnosis falls at least 0.2 absorbency units above the baseline.

(i) Results are assessed visually or in a spectrophotometer, before or after the addition of stopping reagents.

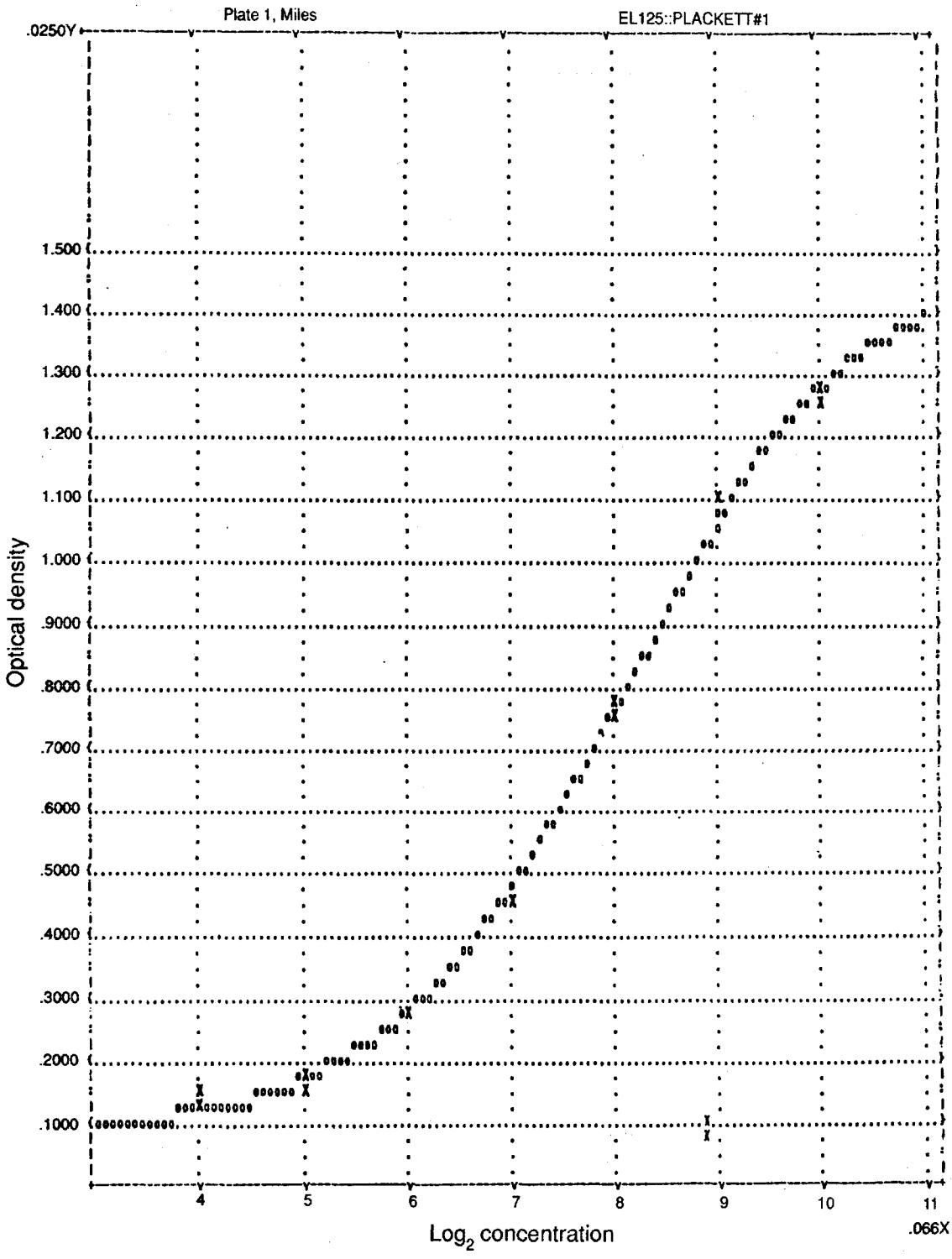

Figure 3. Logistic curve fitted to absorbance readings for dilutions of the New South Wales and anti-*Brucella abortus* reference sera. Ordinate: Absorbance at 450 nm. Abscissa: Log_2 (concentration of antibody units in serum dilution).

6.4. Quality Control and Data Processing

It is important to check the reproducibility of results both for variation between duplicates on each tray and for variations in test sensitivity between trays. This may be done by specifying limits within which absorbance readings given by appropriate dilutions of reference sera should lie. Tests on plates giving values outside these limits can then be repeated until satisfactory results are obtained. Detailed criteria have been developed for use in NSW State Laboratories (Searson, 1985). To allow standardisation between laboratories, results should be expressed in terms of the dilution of the standard serum giving the same absorbance as the test serum under the conditions used. The relationship between the dilution and the level of antibody in 'N units'/mL as defined above is:

N units/mL test serum = (dilution of test serum)/(equivalent dilution of NSW standard serum) x 1024

It is particularly important to check sensitivity and reproducibility in the region of the cut off point: i.e., a 32 000-fold dilution of the reference serum, 64 N units/mL or 6 $\log_2$ units/mL.

It is recommended that the results be expressed on a logarithmic scale. If logs to base 2 are used, the results are related in a simple way to the steps in the two-fold dilution (dln) series used to obtain the data for the standard curve. Thus:

Log_2 (units/mL test serum) = 10 + $\log_2$ (dln of test serum x10/equivalent dln of NSW reference serum).

Table 7 shows values covering the range of interest for tests on unknown sera diluted 200-fold .

The end point is 6 $\log_2$ units/mL. This value, based on extensive studies carried out in New South Wales, is applicable to unvaccinated cattle, the cut-off point for animals known to have been vaccinated with strain 19 being 7 $\log_2$ units/mL (J.E. Searson, pers. comm. 1992).

A plot of absorbance (OD) against the log of antibody concentration for the standard serum dilutions gives a sigmoid curve. The most appropriate equation for curve fitting is that of the four-parameter logistic function (Ukraincik and Piknosh, 1981). Suitable curve fitting programs are well within the capacity of the average microcomputer. A Fortran program for the purpose has been written by Leo Wursthorn of CSIRO Animal Health Laboratory, Parkville. It calculates the curve of best fit for the data obtained from the standard serum dilutions. Fig. 3 shows such a curve for a two-fold dilution series from 2000 to 128 000-fold, together with the observed duplicate OD values for each dilution (X,X). The program then calculates values for unknown samples in the same units, together with estimates of the standard deviation of the assay, and allows corrections to be made for variations in sensitivity due, e.g., to differences in incubation time or temperature. Details are available on request. A number of other programs are available, including some which do not assume a particular form of equation relating optical density to antibody units.

The absorbance values obtained in ELISA tests depend both on antibody concentration and affinity (Lehtonen and Eerola, 1982; Steward and Lew, 1985). The relationship between these quantities are complex, and the distribution of antibody affinities in polyclonal sera may be skewed or even bimodal (Steward and Steensgard, 1983). The ELISA readings given by high and low affinity antibodies are affected differently by changes in serum dilution and in the density of binding sites on microtitre wells. The objective is to establish standard conditions under which the test gives reproducible and consistent 'ELISA values' which can be used as a measure of the probability that the sample came from an infected animal.

Table 7. Calculation of Log_2 units for test serum diluted 1 in 200

Dilution of reference serum (x 10^{-3})	*N units per mL of dilution*	*Log_2 units per mL test serum*
2	1024	10
4	512	9
8	256	8
16	128	7
32	64	6
64	32	5
128	16	4
256	8	3

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8. Appendixes

8.1. Appendix 1 — Kolmer Diluent

8.1.1. Stock Solution

Calcium chloride, $CaCl_2.2H_2O$	24.5 g
Magnesium chloride hexahydrate, $MgCl_2.6H_2O$	101.6 g
Distilled water to	500 mL

Store in refrigerator.

8.1.2. Preparation of Diluent

Sodium chloride, NaCl	17 g
Stock calcium and magnesium solution (see 8.1.1.)	1.25 mL
Sodium azide, NaN_3 (10% w/v, 1.5 mol/L)	0.25 mL
Distilled water to	2 L

Store at room temperature.

8.2. Appendix 2 — Alsever's Solution

Glucose, $C_6H_6O_6$	20.5 g
Sodium chloride	4.2 g
Trisodium citrate, $Na_3C_6H_7.2H_2O$	8.0 g
Citric acid, $C_6H_8O_7$	0.6 g
Distilled (preferably) or deionised water	1 L

Check pH is 6.1.
Dissolve without heating.
Sterilise by filtration or in the autoclave at 115°C for 20 min.

8.3. Appendix 3 — Calibration of Spectrophotometer using Cyanmethaemoglobin Standard

A 3% suspension of sheep erythrocytes prepared as described in 3.2.4. should contain 0.95 g haemoglobin (Hb) per 100 mL. This fact can be utilised in standardising erythrocyte suspensions photometrically. The spectrophotometer can be calibrated to indicate a given concentration of Hb using the cyanmethaemoglobin method, then for routine use this calibration can be converted to the equivalent OD of an erythrocyte suspension lysed in distilled water.

(a) Switch on spectrophotometer, set wavelength at 541 nm, or select the appropriate filter, allow to warm up.
(b) Bring cyanmethaemoglobin standard to room temperature.
(c) Read the OD of the standard on the spectrophotometer, using Drabkin's reagent as blank. (We recommend that Aculute diluent pellets be used for making up the Drabkin's reagent — available from Ortho Diagnostic Division of Ethnor Pty. Ltd, 1 Khartoum Road, North Ryde, NSW, Australia.)
(d) Calculate the OD for a suspension of sheep erythrocytes containing 0.95 g Hb per 100 mL, diluted 1:16 in Drabkin's reagent from the following formula:

OD = [(0.95 x OD of standard x 1000)]/ [Conc. of Standard (mg per 100 mL) x 16]

(e) Prepare a suspension of sheep erythrocytes about (4%).
(f) Mix 1 mL of this suspension with 15 mL Drabkin's reagent, allow to stand for 10 min, then determine the OD.
(g) Calculate the dilution factor required to bring the sheep erythrocyte suspension to the correct concentration (0.95 g haemoglobin per 100 mL) using the formula:

mL to be added = (OD obtained–OD required) x No. of mL to be diluted

(h) Make the necessary dilution and check that this is correct by mixing 1 mL with 15 mL Drabkin's reagent and reading the OD.
(i) Determine the target OD for routine use by lysing 1 mL of the sheep erythrocyte suspension prepared in (h) above in 15 mL distilled water and reading the OD in the spectrophotometer.

8.4. Appendix 4 —Percentage Haemolysis and [y/(100 - y)] for Spectrophotometer with Target OD = 0.5

See Table 7.

8.5. Appendix 5 — Preparation of Crude Lipopolysaccharide from Brucella abortus

ELISA LPS *Brucella abortus* antigen is available from: Dr J.E. Searson, Regional Veterinary Laboratory, Department of Agriculture, Wagga Wagga, NSW 2650, Australia.

B. abortus, strain 19, is grown on a modified glycerol–dextrose-agar medium (GDA-M, see 8.6.) in 80 Roux flasks of 1 L capacity. After four days at 37°C 25 mL of 0.5% (w/v) phenol in distilled water (4°C) is added to each flask. The growth is

Table 7. Percentage haemolysis and $y/(100-y)$ for spectrophotometer with target OD=0.5

Spectro reading	*Per cent Haemolysis*	*y/100-y*	*Spectro reading*	*Per cent Haemolysis*	*y/100-y*
0.45	90	9.0	0.255	51	1.04
0.445	89	8.09	0.25	50	1.0
0.44	88	7.33	0.245	49	0.96
0.435	87	6.69	0.24	48	0.92
0.43	86	6.14	0.235	47	0.89
0.425	85	5.67	0.23	46	0.85
0.42	84	5.25	0.225	45	0.82
0.415	83	4.88	0.22	44	0.79
0.41	82	4.56	0.215	43	0.75
0.405	81	4.26	0.21	42	0.72
0.4	80	4.0	0.205	41	0.69
0.395	79	3.76	0.2	40	0.67
0.39	78	3.55	0.195	39	0.64
0.385	77	3.35	0.19	38	0.61
0.38	76	3.17	0.185	37	0.59
0.375	75	3.0	0.18	36	0.56
0.37	74	2.85	0.175	35	0.54
0.365	73	2.70	0.17	34	0.52
0.36	72	2.57	0.165	33	0.49
0.355	71	2.45	0.16	32	0.47
0.35	70	2.33	0.155	31	0.45
0.345	69	2.23	0.15	30	0.43
0.34	68	2.13	0.145	29	0.41
0.335	67	2.03	0.14	28	0.39
0.33	66	1.94	0.135	27	0.37
0.325	65	1.86	0.13	26	0.35
0.32	64	1.78	0.125	25	0.33
0.315	63	1.70	0.12	24	0.32
0.31	62	1.63	0.115	23	0.30
0.305	61	1.56	0.11	22	0.28
0.3	60	1.5	0.105	21	0.27
0.295	59	1.44	0.1	20	0.25
0.29	58	1.38	0.095	19	0.23
0.285	57	1.33	0.09	18	0.22
0.28	56	1.27	0.185	17	0.20
0.275	55	1.22	0.18	16	0.19
0.27	54	1.17	0.175	15	0.18
0.265	53	1.13	0.17	14	0.16
0.26	52	1.08	0.165	13	0.15

suspended by gentle transverse shaking. The suspension is filtered through several layers of gauze into tared centrifuge bottles and the cells are resuspended in about 1.6 L of cold 0.5% phenol and centrifuged again. The pellets are drained and weighed. Eighty Roux flasks should yield about 200 g wet weight of packed cells.

The pellets are resuspended in distilled water (400 mL per 100 g of packed wet cells) and autoclaved for 20 min at 121°C (Berman *et al.*, 1980). After centrifugation the supernatant is separated and clarified by recentrifugation and/or by passage through a 0.45 µm Millipore filter. It is then made to 0.25 mol/L in sodium hydroxide by adding 1/15 of its volume of 4 mol/L sodium hydroxide, and is heated at 56°C for one hour. It is important that the period of heating is measured from the time the solution reaches 56°C. The solution is cooled, neutralised with glacial acetic acid (CH_3CO_2H) to pH 7.0, and mixed with four volumes of ethanol (C_2H_5OH). After standing at 4°C for 24–48 hours, the flocculent precipitate is collected by centrifugation or filtration (e.g. on a Whatman No. 50 filter paper). It is taken up in a small volume of distilled water and dialysed to remove salts and residual ethanol. The sac contents are centrifuged if necessary to remove any insoluble material. Samples are freeze dried in tared vials to determine the concentration. The stock solution may be stored frozen. Freeze-dried material may be kept in sealed ampules or in a desiccator at room temperature.

8.6. Appendix 6 — Modified Glycerol Dextrose Agar (GDA-M)

Bacto peptone	10 g
Lab Lemco powder	5 g
Sodium chloride	5 g
Bacto agar	25 g
Distilled water	100 g
pH of basal medium	7.0

Heat to dissolve agar then add (to give final concentration):

Dextrose, $C_6H_{12}O_6$	0.1%
Glycerol, $C_3H_8O_3$	2.0%
Sodium metabisulfite, $Na_2S_2O_5$	0.01%
Bacto yeast extract	0.2%
Final pH	7.0

Dispense into Roux flasks and autoclave at 120°C for 30 min.