Technique of the test

The tests may be done in either glass or plastic tubes (8 mm x 50 mm) with rims. Metal agglutination boxes are available for carrying the tubes (see Fig. 17). Each box is open at the top and bottom; on the inside of the 2 side walls, just below the upper edge, are narrow shelves supporting 10 metal strips. Each strip has 10 holes through which the agglutination tubes are suspended by their rims. The strips may be hooked on to the front edge of the box with the tubes on the outside while dilutions of serum are being made; each strip may also be held up in front of a light source to facilitate reading of the results. The boxes fit on top of one another for incubation and storage.

In view of the occasional occurrence of prozone phenomena, at least 5 tubes are normally used for each serum under test. Using an automatic
pipette for preference, 0.8 ml of phenol-saline 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 hours ±1 hour before the results are read. For testing the sera of sheep and goats it is recommended that a 5% sodium chloride solution containing 0.5% of phenol be used, both for making the serum dilutions and for diluting the antigen concentrate. 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 all-glass tuberculin syringe fitted with a needle that has had its bevelled tip removed in such a way 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 different 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 5 standard tubes are prepared by mixing the quantities shown in Table 8.

**Interpretation of results of the European agglutination test**

The results of the agglutination tests should be expressed in International Units (see page 83) and interpreted according to the recommendations contained in the fifth report of the FAO/WHO Expert Committee on Brucellosis, which recommends that in cattle the minimum diagnostic

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level be 100 IU/ml for nonvaccinated animals or those of unknown vaccination status and 200 IU/ml for animals vaccinated with strain 19 at 8 months of age or less. Levels 50% lower than these, i.e., 50 IU/ml for nonvaccinated 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. These standards are no longer accepted by some organizations, notably the European Economic Community. This Community’s regulations require that animals having more than 30 IU/ml of agglutinating antibody be regarded as having failed the test.

In humans, high or rising titres are presumptive evidence of *Brucella* infection. Low titres or negative reactions, however, do not exclude it. Low titres are quite significant when the mercaptoethanol test shows the presence of 7S immunoglobulin.

**The international unitage system for reporting agglutination results**

Antigens for the tube agglutination test used in different countries may vary in density; this factor, and also differences in the technique of performing the test, often result in different titres being obtained with the same positive serum. To permit the results given by different antigens and methods to be equated, the International Standard Anti-*Brucella abortus* Serum (ISAbS) was established. The use of this standard serum allows the results of agglutination tests to be reported in terms of International Units no matter what antigen or method was used for carrying out the test.

When the stocks of the first ISAbS became exhausted, a replacement standard, the second ISAbS, was established in 1968. The International Unit was then defined as the activity contained in 0.09552 mg of the second ISAbS. Each ampoule of the second ISAbS contains 1000 IU. This serum was prepared from a cow that had been experimentally infected with *B. abortus* biotype 1, strain 544 (reference strain) and contains antibodies that are almost entirely IgG.

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1 Available from: the FAO/WHO Collaborative Centre for Brucellosis, Central Veterinary Laboratory, New Haw, Weybridge, England.
In order to express in International Units the results obtained with a particular agglutinating antigen, using a particular method, it is necessary first to establish the titre obtained when the ISAbS is tested by this antigen and method.

In practice the contents of one ampoule of the ISAbS are reconstituted in 1 ml of distilled water, giving a solution containing 1000 IU in 1 ml. This serum is then tested by the routine technique of the laboratory concerned to establish the dilution of ISAbS that gives 50% agglutination. It may be necessary to make dilutions intermediate between those normally employed, in order to establish exactly the dilution that gives 50% agglutination. A simple calculation now suffices to establish the titre in International Units of any serum that may be tested, as illustrated by the following 2 examples.

By definition the ISAbS contains 1000 IU per ampoule. Therefore, using an antigen, that gives a titre of 1:500 with the ISAbS, a serum giving a titre of 1:40 contains $1000 \times \frac{40}{500} = 80$ IU/ml. Table 9 gives International Units for various endpoints obtained in this system.

The ISAbS has a titre of 1:1000 when tested by the USDA tube agglutination test, therefore, titres obtained in the USDA test are equal to International Units, e.g., a serum giving + at 1:25 (see Table 5, page 74) contains 25 IU/ml.

It is recommended that, in publications, results of tube agglutination tests should always be given in IU/ml.

There is some evidence that in infections with B. melitensis biotype 1, higher titres can be obtained by using homologous (B. melitensis) antigens than by using the heterologous (B. abortus) antigen, when both have been standardized against the ISAbS. The differences are less marked in some animal species than in others. In areas where infection due to B. melitensis biotype 1 is the predominant type of brucellosis present, agglutinating antigens may, with advantage, be prepared from an attenuated smooth culture of B. melitensis biotype 1, such as the Rev. 1 vaccine strain (see page 140).

As stocks of the ISAbS are limited, it is necessary that countries should prepare their own national standards. Methods for preparing such standards follow.

**Preparation of a standard dried serum**

Essentially, all that is necessary is to obtain an anti-B. abortus serum of the right titre and to dry it in such a way that its titre is preserved and that it can be reconstituted easily. Drying produces practically no reduction in titre if properly carried out. It is desirable that the standard serum should contain antibodies that are entirely IgG, i.e., the serum chosen
<table>
<thead>
<tr>
<th>Final dilution of serum</th>
<th>End-point reading</th>
<th>IU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:10</td>
<td>+</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>++</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>+++</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>++++</td>
<td>27</td>
</tr>
<tr>
<td>1:20</td>
<td>+</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>++</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>+++</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>++++</td>
<td>53</td>
</tr>
<tr>
<td>1:40</td>
<td>+</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>++</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>+++</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>++++</td>
<td>106</td>
</tr>
<tr>
<td>1:80</td>
<td>+</td>
<td>134</td>
</tr>
<tr>
<td></td>
<td>++</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td>+++</td>
<td>195</td>
</tr>
<tr>
<td></td>
<td>++++</td>
<td>212</td>
</tr>
<tr>
<td>1:160</td>
<td>+</td>
<td>268</td>
</tr>
<tr>
<td></td>
<td>++</td>
<td>320</td>
</tr>
<tr>
<td></td>
<td>+++</td>
<td>372</td>
</tr>
<tr>
<td></td>
<td>++++</td>
<td>424</td>
</tr>
<tr>
<td>1:320</td>
<td>+</td>
<td>536</td>
</tr>
<tr>
<td></td>
<td>++</td>
<td>640</td>
</tr>
<tr>
<td></td>
<td>+++</td>
<td>744</td>
</tr>
<tr>
<td></td>
<td>++++</td>
<td>848</td>
</tr>
<tr>
<td>1:510</td>
<td>+</td>
<td>1072</td>
</tr>
<tr>
<td></td>
<td>++</td>
<td>1190</td>
</tr>
<tr>
<td></td>
<td>+++</td>
<td>1488</td>
</tr>
<tr>
<td></td>
<td>++++</td>
<td>1692</td>
</tr>
</tbody>
</table>

*Morgan, et al. (1971) using antigen standardized to give 50% agglutination with a 1:500 dilution of the ISAbS.*

should have the same titre to the mercaptoethanol test as it has to the ordinary agglutination test.

A bovine animal whose serum meets the requirement described above should be bled and a large volume of serum collected—sufficient to last for several years. The titre of this serum is adjusted by dilution with a negative bovine serum until it is exactly equal to the titre of the ISAbS. It is dispensed in 1-ml quantities and freeze-dried.

An alternative method of producing a national standard serum is to select a cow infected with *B. abortus* biotype 1 that has a titre equal to or higher than that given by the ISAbS and due entirely to the presence of IgG antibodies. This animal is bled, the serum is separated, filtered, and freeze-dried in 1-ml quantities.

Subsequently, the contents of 1 ampoule of this national standard serum are reconstituted in 1 ml of distilled water and this solution is subjected to a tube agglutination test with the antigen and method used in
the laboratory concerned. This test is done in duplicate with the ISAbS. It may be necessary with the national standard serum to make dilutions intermediate between those normally used, to establish exactly the dilution of the national standard serum that gives 50% agglutination in the system being used. In this way the ratio between the titres given by the ISAbS and the national standard is established, and this is the factor indicating how much the national standard needs to be diluted to produce a solution having the same number of International Units per ml as 1 ampoule of ISAbS reconstituted in 1 ml of diluent; e.g., if the above series of titrations yields a titre of 1:500 for the ISAbS and 1:550 for the national standard, then the latter will need to be reconstituted in 550/500 = 1.1 ml to make a solution equal in potency to the ISAbS reconstituted in 1 ml of diluent. To avoid errors in pipetting, it would be more practical in this case to reconstitute 1 ampoule of the national standard in 11 ml of phenol-saline, which would correspond to a dilution of 1:10 of the ISAbS; further dilutions could be made from this solution.

Subsequently, the national standard serum, diluted according to its dilution factor, is used for standardizing batches of locally produced antigen, or as a control serum in routine tests. Where this system has been adopted occasional rechecking of the national standard serum against the ISAbS should be carried out, perhaps annually or every second year.

The complement fixation test

The complement fixation test is widely used for the diagnosis of brucellosis in cattle, sheep, and goats and is of particular value as a means of distinguishing between serological reactions resulting from vaccination with living vaccines and those produced by natural infection.

The most commonly used technique for complement fixation involves cold fixation at 0-4°C for 14-18 hours. This method is completely satisfactory for testing human sera against brucellosis, but in the field of bovine brucellosis many workers prefer to use warm fixation for a shorter period of time, usually half an hour, at 37°C.

Several factors influence the choice of method:

1. It is generally considered that the warm fixation method is more convenient to operate.

2. The anti-complementary activity of cattle, sheep, and goat sera is greater than that of human sera, but where the warm fixation method is employed these animal sera can be successfully inactivated by heating to 58°C, whereas they must be heated to at least 60°C before cold fixation.

3. Higher titres are obtained with cold fixation.
4. Prozones occur frequently, sometimes to high titre, with warm fixation, rendering difficult the adaptation of the system to a 1- or 2-tube procedure. Prozones, however, are less evident with cold fixation.

In this monograph 2 complement fixation techniques are described; the first, in which either cold or warm fixation may be used in macro (1-ml) or micro (0.1-ml) volumes, includes also a 1-tube test. It is called the composite complement fixation technique, is based on the method described in Public Health Monograph No. 74 (1965) of the US Public Health Service, and uses five 50% haemolytic units of complement. The second technique described is a modified form of that of Hill (1963), which is now widely used in the diagnosis of bovine brucellosis.

Those procedures that are common to both techniques, i.e., preparation of glassware and plastic plates, diluents, collection and storage of sheep erythrocytes, and preparation of haemolysin and complement, are described first.

Cleaning and preparation of glassware and plastic plates

After use, glassware and plastic plates should be washed in detergent solution to remove all reagents and then placed in dilute chromic acid cleaning fluid for 18 hours (potassium dichromate 20 g; sulfuric acid 76.6 ml, and distilled water to 1 litre). They 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 and plastic plates in the incubator at 37° C.

Diluents

An isotonic salt solution, preferably buffered, containing calcium and magnesium ions, is used for preparing all solutions and suspensions in complement fixation work; a choice of 3 diluents is given.

Normal saline solution with magnesium and calcium

This is the simplest diluent that is suitable for use in complement fixation tests. It consists of a 0.85% solution of sodium chloride to each litre of which is added 1.2 ml of a 0.416 mol/litre solution of magnesium chloride and 1.2 ml of a 0.125 mol/litre solution of calcium chloride. The stock solutions of the magnesium and calcium salts should be stored in the refrigerator. Both these salts are very hygroscopic and it is recommended that the following procedure be used for making up the stock solutions. Arbitrary quantities of magnesium chloride hexahydrate (MgCl₂·6H₂O) and calcium chloride dihydrate (CaCl₂·2H₂O) are transferred to preweighed glass-stoppered weighing bottles and the quantity of chemical in each bottle is weighed. The salts are then dissolved in distilled
water to a final volume of 11.8 ml per g of MgCl₂·6H₂O and to a final volume of 54.4 ml per g of CaCl₂·2H₂O. This is done by pouring the powder from the weighing bottles into measuring cylinders and rinsing the weighing bottles with successive small quantities of distilled water to ensure that all the salt becomes incorporated in the solution, which is then made up to the desired volume with distilled water. If sodium azide, at the rate of 0.8 g per litre, is added to control bacterial contamination, the diluent may be stored in bulk at room temperature.

*Barbital-buffered saline solution*

This is a very commonly used diluent for complement fixation; it is efficient, but takes longer to prepare than do the other 2 diluents. The following procedure is taken from Kabat & Mayer (1961).

(a) Prepare a stock solution containing 1 mol/litre magnesium chloride and 0.3 mol/litre calcium chloride, e.g., 9.5 g of anhydrous magnesium chloride and 3.7 g of anhydrous calcium chloride made up to 100 ml in distilled water. Pass through a Seitz filter and store in the refrigerator in small amounts.

(b) Dissolve 85 g of sodium chloride and 3.75 g of sodium 5,5-diethylbarbiturate (barbital sodium) in about 1400 ml of distilled water.

(c) Dissolve 5.75 g of 5,5-diethylbarbituric acid (barbital) 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), and add distilled water to make a final volume of 2000 ml. This is the concentrated barbital buffer solution, which should be stored in a refrigerator.

(e) For use, 1 part of the concentrated buffer solution is mixed with 4 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 4 parts of sterile 0.04% gelatin solution rather than in distilled water.

Tablets are available commercially¹ that produce a barbital-buffered salt solution similar to the above when dissolved in distilled water.

*Triethanolamine-buffered saline (TBS)*

This solution is efficient in use and is convenient to prepare.

A tenfold concentrated solution is made up and may be stored in the refrigerator for a number of months. For use, 1 part of the concentrated

buffer is added to 9 parts of distilled water; the diluted solution should not be kept for more than 1 week and should be stored in the refrigerator.

Formula for the tenfold concentrated solution:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>sodium chloride</td>
<td>75 g</td>
</tr>
<tr>
<td>hydrochloric acid 1 mol/litre</td>
<td>177 ml</td>
</tr>
<tr>
<td>triethanolamine</td>
<td>28 ml</td>
</tr>
<tr>
<td>magnesium chloride (4.16 mol/litre solution)</td>
<td>1.2 ml</td>
</tr>
<tr>
<td>calcium chloride (1.25 mol/litre solution)</td>
<td>1.2 ml</td>
</tr>
<tr>
<td>distilled water to</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

Dissolve the sodium chloride in about 600 ml of distilled water in a 1-litre volumetric flask; then add the other reagents in the order given. Triethanolamine is a highly viscous liquid and needs to be accurately measured; this may be done by pouring it into a measuring cylinder down a glass rod or adding from a pipette in such a way that the triethanolamine does not touch the walls of the cylinder above the 28-ml mark; alternatively, weigh the triethanolamine (28 ml weigh 31.45 g) in a beaker. The density of different batches may vary slightly, necessitating an adjustment in the weight required. Whichever method is adopted, the container holding the measured amount of triethanolamine must be thoroughly rinsed with the solution in the volumetric flask to ensure that all the triethanolamine is incorporated in the diluent. The stock solutions of magnesium and calcium chlorides are made up as described for the first diluent on page 87, but 10 times more concentrated; i.e., 10 g of MgCl₂.6H₂O make 11.8 ml of 4.16 mol/litre stock solution and 10 g of CaCl₂.2H₂O make 54.4 ml of 1.25 mol/litre stock solution.

The pH of the diluted solution should be 7.3–7.4 at 20°C; each new lot of tenfold concentrated diluent should be checked, after diluting a sample, before it is used.

**Collection and storage of sheep erythrocytes**

One or more sheep, known to produce erythrocytes of a consistently satisfactory level of sensitivity, should be chosen and subsequently used exclusively. Blood is withdrawn under aseptic conditions into an equal volume of Alsever’s solution and thoroughly mixed. Alsever’s solution is made up as follows:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>glucose</td>
<td>18.66 g</td>
</tr>
<tr>
<td>sodium chloride</td>
<td>4.18 g</td>
</tr>
<tr>
<td>sodium citrate</td>
<td>8.00 g</td>
</tr>
<tr>
<td>citric acid</td>
<td>0.55 g</td>
</tr>
<tr>
<td>distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

The solution may be autoclaved or passed through a Seitz filter. The sheep’s blood so preserved is stored aseptically in screw-capped bottles in the
refrigerator and not used until at least 5 days after collection; thereafter it may be used for up to 6 weeks, provided it does not become contaminated.

**Haemolysin (amboceptor)**

This is a serum containing a high level of antibody against sheep erythrocytes. When this antibody is combined with erythrocytes in suspension the erythrocytes are said to be sensitized, i.e., in the presence of free complement they will lyse. The haemolysin is titrated as described later, on pages 92 and 106, depending on the complement fixation technique being used.

The haemolysin is usually prepared in rabbits. Details of the technique of producing haemolysin may be found in most textbooks giving serological techniques (e.g., Campbell et al., 1963; or Cruickshank, 1965). Haemolysin is also available commercially, usually in liquid form preserved in an equal volume of glycerol.

**Preparation and storage of complement**

At least 4 guineapigs should be bled, the serum separated as soon as practicable from the clot, and pooled to produce complement. Adult guineapigs 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. The complement may be stored frozen at $-40^\circ$ C or below; where facilities exist, storage in liquid nitrogen is both efficient and convenient. Complement may be freeze-dried or dried complement purchased; it should be stored in the refrigerator or freezer even when dried.

Where adequate facilities for drying or freezing complement are not available, it may be preserved by Richardson's method and when so preserved will maintain its titre for about 6 months if stored at $0-4^\circ$ C. Even at room temperature, loss of titre is not rapid.

Two stock solutions that keep indefinitely are used:

**Solution A**

<table>
<thead>
<tr>
<th>Substance</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boric acid ($H_2BO_3$)</td>
<td>0.93 g</td>
</tr>
<tr>
<td>borax ($Na_2B_4O_7\cdot10H_2O$)</td>
<td>2.29 g</td>
</tr>
<tr>
<td>sorbitol ($C_6H_{12}O_6\cdot\frac{1}{2}H_2O$)</td>
<td>11.74 g</td>
</tr>
<tr>
<td>saturated sodium chloride solution to</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

**Solution B**

<table>
<thead>
<tr>
<th>Substance</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>borax</td>
<td>0.57 g</td>
</tr>
<tr>
<td>sodium azide ($NaN_3$)</td>
<td>0.81 g</td>
</tr>
<tr>
<td>saturated sodium chloride solution to</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

To preserve complement, mix 8 parts of guineapig serum with 1 part of Solution B, followed by 1 part of Solution A. Before use, each 1 ml of preserved complement is added to 7 ml of distilled water to restore tonicity;
this produces a 1:10 dilution of complement; further dilutions are made in complement fixation diluent.

The titration of complement is described on pages 95 and 106.

The composite complement fixation technique

The system to be described utilizes the plateau technique for titrating haemolysin to determine the amount of haemolysin required to provide an optimally sensitized erythrocyte suspension and five 50% haemolytic units of complement. Either warm or cold fixation may be employed in either macro (1-ml) or micro (0.1-ml) volumes. The single-tube test to be described requires cold (overnight) fixation because of the occurrence of prozone phenomena with warm fixation.

The system is extremely sensitive; reproducible results will only be obtained when care is taken in handling the various components used and in the preparation of glassware and reusable plastic plates. The titrations may at first sight appear complicated, but after a little practice are as rapidly and easily performed as older methods. Tubes are always used for titrations.

Preparation of the sheep erythrocyte suspension

An appropriate quantity (up to 10 ml) of sheep erythrocytes stored in Alsever's solution (page 89) 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 thin layer of white cells that overlays the deposit. The erythrocytes are resuspended in fresh diluent and the centrifuging repeated. For the third and final centrifuging the erythrocytes are resuspended in about 15 ml of diluent and centrifuged in a graduated tube at exactly 1000 g for 10 minutes. The volume of the deposit is then read and after discarding the supernatant each ml of deposit is suspended in 32.33 ml of diluent to make a 3% suspension.

If a suitable photometric apparatus is available the erythrocyte suspension may be standardized by taking a given quantity of a denser suspension and measuring the optical density (O.D.) of the resulting haemoglobin solution at the wavelength setting (541 nm) or with the filter appropriate to haemoglobin estimation; this allows calculation of the factor by which the denser suspension needs to be diluted to produce an exactly 3% suspension of erythrocytes. It is necessary first to calibrate the particular machine being used by determining the O.D. produced by a 3% erythrocyte suspension appropriately laked. This is called the target O.D.

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1 Several instruments are suitable: The Coleman Junior, the Bausch & Lomb Spectronic and the Beckman spectrophotometers; the Klett-Summerson photoelectric colorimeter. The MRC grey wedge haemoglobinometer (Keeler Optical Products, 39 Wigmore St, London, England) is a small machine that can be held in the hand.
A 3% erythrocyte suspension for calibrating the photometer may be prepared by one or more of the following methods: (a) the packed-cell volume method described above; (b) by preparing a suspension containing 7 x 10^6 erythrocytes per ml using an electronic cell counter, or (c) by preparing a suspension of erythrocytes containing 0.95 g of haemoglobin per 100 ml determined by the cyanmethaemoglobin method as described in textbooks of haematology. Whichever method is used for preparing the erythrocyte suspension, the suspension is made by adding 1 ml to 15 ml of distilled water and the O.D. is determined. The procedure needs to be repeated several times and an average taken to determine the target O.D. (On a Bausch & Lomb Spectronic 20 set at wavelength 541 nm and using the standard sample tube of 11.7 mm internal diameter, the target O.D. is 0.5, though there may be some variation from one machine to another.)

In the routine preparation of the erythrocyte suspension by the photometric method, the deposit after the third centrifuging is suspended in about 27 times its volume of diluent to make the denser erythrocyte suspension; after thorough mixing, 1 ml of this suspension is made by mixing with 15 ml of distilled water and the O.D. is determined. The factor by which the denser suspension needs to be diluted is calculated according to the following example, which takes 0.5 as the target O.D. If the initial O.D. is 0.61, then 0.61 - 0.5 or 0.11 ml of diluent needs to be added to each 0.5 ml of the denser suspension (or, say 11 ml to 50 ml) to produce the 3% erythrocyte suspension required for the test. Each final suspension must be checked by taking a sample before use. The erythrocyte suspension, if stored at about 3°C, may be used for up to 24 hours after preparation.

Titration of haemolysin

It is convenient to prepare a 1:100 dilution of haemolysin (see page 90) in complement fixation diluent and store frozen in aliquots. Titrate as follows:

1. Prepare a 3% suspension of sheep erythrocytes.
2. From the 1:100 stock dilution of haemolysin prepare the following range of dilutions in complement fixation diluent: 1:1000, 1:1500, 1:2000, 1:3000, 1:5000, 1:8000, and 1:16000.
3. Add 1 ml of each haemolysin dilution to 1 ml of 3% sheep erythrocytes while gently agitating the erythrocyte suspension, then leave the mixtures at room temperature for 15 minutes to allow sensitization of the erythrocytes to occur, agitating the tubes from time to time.

The rest of the procedure is done in duplicate to minimize pipetting errors and to provide a volume sufficient to give a reading in the photometer.

4. To each of a duplicate series of 7 tubes add 0.5 ml of diluent and 0.25 ml of complement diluted in such a way that it will produce approxi-
mately 70–80% haemolysis with the more concentrated haemolysin dilutions (with good quality complement a 1:400 dilution is usually satisfactory).

5. Add 0.25 ml of one of the 7 sensitized erythrocyte suspensions to each pair of tubes containing complement and diluent and mix the contents thoroughly.

6. Incubate the tubes for half an hour in a water bath at 37° C, with gentle shaking after 15 minutes.

7. Remove the tubes from the water bath, add 1 ml of cold diluent to each, then centrifuge the tubes to deposit any erythrocytes remaining unlysed.

8. Pour off the supernatant from each tube, amalgamating the contents of each pair of tubes, and read the O.D.s in a photometer; alternatively, the percentage haemolysis may be estimated by comparison with haemolysis standards without the addition of cold diluent.

9. The O.D. corresponding to 100% haemolysis in this system is the same as the target O.D. used in adjusting the erythrocyte suspension as described on page 92 (in the example using the Spectronic 20 it would be 0.5).

10. Calculate the percentage haemolysis for each dilution in the titration; e.g., in the above system, using the Spectronic 20, the O.D. produced by 100% lysis is 0.5 and therefore a tube showing an O.D. of 0.21 would have $0.21 \times 100/0.5 = 42\%$ haemolysis.

11. Plot the percentage haemolysis given by each dilution on graph paper marked out as shown in Fig. 18. To calibrate the abscissa, measure an arbitrary distance, say 6 scale divisions from the left-hand extremity and place a point here representing the 1:1000 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 into 1000; e.g., the point for the 1:2000 dilution is placed 1000/2000 or half-way along the line, the 1:8000 dilution 1000/8000 or one-eighth of the way along, and so on.

The percentage haemolysis is marked linearly along the ordinate.

12. The points corresponding to the percentage of haemolysis produced by each dilution of haemolysin are marked on the paper and connected by a line. Percentage haemolysis at first rises rapidly corresponding to increased haemolysin present, but later the graph flattens to form a plateau, since beyond a certain point an increase in the quantity of haemolysin present produces practically no increase in the percentage of haemolysis. The erythrocytes are then said to be optimally sensitized.
13. The optimal dilution for use in the test is determined by taking the second point along the plateau. In Fig. 18 the plateau may be taken to begin at 1:2000, and therefore in further steps in the complement fixation test the haemolysin is diluted 1:1500. The selection of the quantity of haemolysin to use in the test is not critical so long as an ample amount is used, as it forms a fixed point against which the amount of complement to be used is determined accurately.

Haemolysis standards

As an alternative to using a photometric device, a reasonably accurate assessment of the degree of haemolysis present in a given tube may be obtained by use of haemolysis standards.

A suspension of sheep erythrocytes is mixed with a solution of laked erythrocytes to simulate various degrees of haemolysis.

1. The sheep erythrocyte suspension is prepared by adding 1 ml of a 3% suspension of erythrocytes to 7 ml of diluted diluent.
2. The laked erythrocyte (haemoglobin) solution is prepared by mixing 1.0 ml of the 3% erythrocyte suspension with 6.3 ml of distilled water, shaking until complete lysis has occurred, then adding 0.7 ml of tenfold concentrated diluent to restore tonicity. (Where a fivefold concentrated barbiturate buffer is used, 1.0 ml of erythrocytes should be laked by adding 5.6 ml of distilled water and the tonicity restored with 1.4 ml of concentrated buffer.)

The standards representing 0–100% haemolysis are then prepared by mixing the erythrocyte suspension (Step 1) with the haemoglobin solution (Step 2) in the proportions shown in Table 10.

<table>
<thead>
<tr>
<th>TABLE 10. PREPARATION OF HAEMOLYSIS STANDARDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage haemolysis</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>Sheep erythrocyte suspension (ml)</td>
</tr>
<tr>
<td>1.0</td>
</tr>
<tr>
<td>Laked erythrocyte solution (ml)</td>
</tr>
<tr>
<td>0</td>
</tr>
</tbody>
</table>

The haemolysis standards are made up just before the haemolytic system is added in the titration or diagnostic test for which they are to act as standards. The standards are incubated and centrifuged in the same way as the test. (Cold diluent is not added to titration tubes when these are to be compared with haemolysis standards.)

**Titration of complement**

The quantity of complement required to lyse 50% of optimally sensitized erythrocytes is determined. A master dilution of complement, 10 times stronger than that required for the titration, is prepared and stored in the refrigerator. The master dilution should be made in diluent at refrigerator temperature (2–3°C) and stored at this temperature. With good quality complement a master dilution of 1:40 is made, and for the titration a small quantity is further diluted in 9 volumes of diluent to produce a 1:400 dilution. With complement of different potency the dilution factors are determined by trial.

The procedure for the titration is as follows:

1. Prepare sensitized erythrocytes by mixing equal volumes of 3% erythrocytes and haemolysin diluted as determined in the previously described haemolysin titration. Allow the mixture to stand at room temperature for 15 minutes, mixing from time to time.
2. Prepare master and titration dilutions of complement.

3. The test is done in twice the standard volume to minimize errors in pipetting and to produce a total volume large enough to give a reading on the photometer. Arrange 4 suitable tubes in a rack and add the reagents in the quantities and the order shown in Table 11.

<table>
<thead>
<tr>
<th>Complement 1:400 (ml)</th>
<th>0.3</th>
<th>0.4</th>
<th>0.5</th>
<th>0.6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluent (ml)</td>
<td>1.2</td>
<td>1.1</td>
<td>1.0</td>
<td>0.9</td>
</tr>
<tr>
<td>Sensitized erythrocytes (ml)</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

4. The contents of each tube are mixed by gentle agitation, then incubated in a water bath at 37°C for 30 minutes, during which time they are agitated once—at 15 minutes. After the incubation has been completed, the tubes are removed from the water bath, 2.0 ml of cold diluent are added to each tube, and the tubes centrifuged to deposit the unlysed cells. The supernatants are then poured off and their various O.D.s determined in the photometer or by comparison with haemolysis standards (in the latter case the cold diluent is not added). The percentage of haemolysis is calculated as before (page 93, paragraph 10).

5. The values for the expression $y/(100−y)$ are now calculated for each dose of complement, $y$ being the percentage of haemolysis (only tubes showing between 10% and 90% haemolysis are taken into consideration). The calculation is made as in the examples given in Table 12.

<table>
<thead>
<tr>
<th>Dose of complement</th>
<th>Optical density (OD)</th>
<th>Percentage haemolysis (y)</th>
<th>$y/(100−y)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>0.065</td>
<td>13</td>
<td>0.15</td>
</tr>
<tr>
<td>0.4</td>
<td>0.235</td>
<td>47</td>
<td>0.09</td>
</tr>
<tr>
<td>0.5</td>
<td>0.36</td>
<td>72</td>
<td>2.6</td>
</tr>
<tr>
<td>0.6</td>
<td>0.445</td>
<td>80</td>
<td>8.1</td>
</tr>
</tbody>
</table>

To reduce the amount of calculation required, a table should be drawn up showing (a) percentage haemolysis and (b) $y/(100−y)$ for each photometer reading in the range 10–90% haemolysis.
6. The values for the expression \( y/(100-y) \) are plotted for each dose of complement on log/log paper (a simplified form is shown in Fig. 19) and a slope is constructed by connecting the points by a straight line with as good a fit as possible. The point where the slope crosses the "1" line indicates the 50% haemolytic dose (CH50) and this may be read off from the ordinate (see the broken line in Fig. 19).

FIG. 19. TITRATION OF COMPLEMENT

7. A simple calculation suffices to indicate the dilution factor required for the diagnostic test. In our example, 0.42 ml of a 1:400 dilution contains 1CH50; therefore 5×0.42 ml, or 2.1 ml, of this dilution would contain 5CH50 and to calculate the dilution that would contain this amount of complement in 0.5 ml (here we are using double volumes) the following equation is used, where \( X \) is the dilution factor required: 400/2.1 = \( X/0.5 \) or \( X = 95 \). The fact that smaller volumes are used in the diagnostic test does not affect this dilution factor, since the proportions of each reagent remain the same. The 1:40 dilution of complement now being stored in the refrigerator should, therefore, be diluted 95.40 or 2.37 times to bring it to the 1:95 dilution required for use in the diagnostic test; i.e., each 1 ml of the 1:40 dilution is added to 1.37 ml of diluent. The 1:40 dilution will maintain its potency throughout the working day if stored as described. When cold fixation is being used, it is advisable to use a complement dilution 10% more concentrated than that calculated by the above method.

8. The validity of the slope of the line is now examined. A horizontal line 10 cm long (line A-B in Fig. 19, reduced in size) is drawn with its left-hand extremity touching the slope at a convenient point. From the right-hand end of this line at B, a vertical line is extended up to the point where it meets the slope at C. With a satisfactory slope the length of the vertical line (B-C) should be 20 mm±10%, i.e., the vertical line should not be more than 22 mm and not less than 18 mm long. Though complement giving steeper or flatter
slopes may give satisfactory results in the diagnostic test, experience has shown that accurate and reproducible results are more likely to be obtained if complements satisfying these criteria are used.

**Titration of antigen**

A particulate antigen composed of heat-killed *B. abortus* cells, such as the concentrated suspension used for the agglutination test, is suitable. The most sensitive dilution of antigen is determined in a block titration against one or more anti-*Brucella* sera of moderate or low titre. A typical titration result is shown in Table 13, the results are expressed as the percentage of haemolysis found in each tube.

<table>
<thead>
<tr>
<th>Antigen dilution</th>
<th>Dilutions of serum</th>
<th>Anti-complementary controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:5</td>
<td>1:10</td>
</tr>
<tr>
<td>1:100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1:200</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1:400</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1:800</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1:1600</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Diluent in lieu</td>
<td>100</td>
<td>—</td>
</tr>
</tbody>
</table>

The serum is inactivated (see page 99), and then a series of doubling dilutions is made in a sufficient volume for each dilution to supply 0.25 ml to at least 6 tubes; 0.25 ml of the appropriate dilution is dispensed into the tubes in the vertical rows, leaving the anti-complementary control tubes empty. The antigen dilutions are now prepared from the *B. abortus* agglutination antigen concentrate and then dispensed in 0.25-ml volumes into the tubes in the horizontal rows, including the anti-complementary controls. The last horizontal row receives 0.25 ml of diluent in the first and last three tubes in lieu of antigen; the first tube serves to detect any anti-complementary action in the serum and the last three are complement controls. Five CH₉₀ of complement, as determined in the previously described complement titration, contained in 0.25 ml are now placed in all the tubes containing serum and in the first vertical row of anti-complementary controls. The row headed 2CH₉₀ receives 0.1 ml of the complement dilution, and that headed 1CH₉₀ receives 0.05 ml. To bring the volumes in these control tubes up to the volume in the others, 0.25 ml of diluent is placed in each tube in the first vertical row, 0.4 ml in each tube of the second vertical row, and 0.45 ml in each tube of the last vertical row.

The tubes are now placed in the water bath at 37° C for 30 minutes or in the refrigerator for 14–16 hours, depending on the type of fixation to be used in the diagnostic test. After this period for fixation, 0.25 ml of sensitized erythrocytes is placed in each tube and the tubes are placed in the water bath at 37° C for 30 minutes, after which time the results are read.
To read the result the tubes are centrifuged to deposit any unlysed cells and the percentage of haemolysis is determined by comparing the supernatants with colour standards or by use of a photometer. In the latter case, in order to provide an adequate volume for the photometer it will probably be necessary to use double the volumes shown above, then to add an equal volume (2 ml) of cold diluent to each tube before centrifuging. The most sensitive dilution is the dilution chosen for use in the diagnostic test (1:400 in Table 13); where 2 dilutions prove to be approximately equal in sensitivity, the more concentrated is used in the diagnostic test, as the more concentrated the antigen the less the likelihood of prozones occurring, especially with fixation at 37° C. In the example given in Table 13 an antigen dilution of 1:200 would be preferred for warm fixation.

A killed suspension of *B. abortus*, such as that recommended for use as antigen in this test, does not normally possess any anti-complementary activity. In the anti-complementary controls, therefore, the dilution chosen for use in the diagnostic test would show 100% haemolysis with the 5CH₅₀ control and at least 70% with 2CH₃₀. The serum control tube (1:5 dilution of serum with diluent in lieu of antigen) must show 100% lysis. Sera exhibiting anti-complementary activity at this dilution are not suitable for use in the titration.

**Inactivation of sera**

In addition to the natural anti-complementary activity present in serum, certain conditions, particularly bacterial contamination, may produce an added degree of anti-complementary activity. Haemolysis in serum has little, if any, anti-complementary effect. The anti-complementary activity due to the presence of bacteria may be greatly reduced by high-speed centrifugation of the serum.

The natural anti-complementary activity is removed from sera by heating for 30 minutes in a water bath. Human serum may be inactivated at 56° C. In the case of sera from cattle, sheep, and goats, if these are to be tested by the warm (37° C) method, the temperature of inactivation should be 58° C; if they are to be tested by cold fixation, it should be 62° C. Serum may be inactivated in concentrated form or after dilution.

**The diagnostic test**

*The standard (macro) technique*

The total volume used is 1 ml, and either tubes or plastic haemagglutination plates may be used. The number of dilutions of each serum sample to be tested will depend on circumstances; with warm (37° C) fixation, serum dilutions up to at least 1:128 are required because of the prevalence of prozones. For the purpose of description we will assume that 6 dilutions will be tested and that the test will be done in tubes; a seventh
tube to serve as a control on the anti-complementary action of each serum will also be required.

1. Dilute each serum sample to the dilution required for the first tube in the diagnostic test—normally 1:4 or 1:5. A total quantity of 1 ml of the dilution is convenient. Inactivate (see above).

2. Arrange the tubes in rows, 1 row for each serum to be tested, and with an automatic pipette add 0.25 ml of diluent to each tube, excepting the first of each row but including the serum control tube.

3. With a tuberculin syringe (see page 82, paragraph 1) take up 0.75 ml of the inactivated first serum dilution, place 0.25 ml in the first and last tubes (tube No. 7—the serum control); then place the remaining 0.25 ml in the second tube, mixing with the 0.25 ml of diluent already there, and transfer 0.25 ml of the mixture to tube No. 3, and so on up to tube No. 6, from which, after mixing, 0.25 ml is discarded.

4. A standard serum with a low positive titre should be included with each set of tests. This serum should be stored in aliquots, either frozen or freeze-dried.

5. Add 0.25 ml of appropriately diluted antigen to all the tubes except the serum control tubes.

6. Add 0.25 ml of appropriately diluted complement (see page 97, paragraph 7) to all the tubes. If cold fixation is being used, it is advisable to use complement 10% more concentrated. The diluted complement should be kept cool while it is being dispensed, especially if cold fixation is being used.

7. As a check on the degree of complement activity present in the system, 4 control tubes are set up, each containing 0.05 ml of complement (I.CH₃) and 0.7 ml of diluent; the complement should be carefully dispensed with a 0.1-ml pipette. These controls should be done in tubes even if the rest of the test is done in plastic plates.

8. Keep all the tubes (tests and controls) either at 0–4°C (refrigerator temperature) for 14–18 hours or at 37°C in the water bath for 30 minutes.

9. Prepare sensitized sheep erythrocytes by mixing equal volumes of appropriately diluted haemolysin and of 3% sheep erythrocytes. In the case of cold fixation overnight this will be done next morning. The haemolysin is added to the erythrocyte suspension while the latter is being gently agitated; mix thoroughly by pouring from one container to another and back again. Allow to remain at room temperature for 15 minutes, mixing from time to time; then, as soon as the fixation period is completed, add 0.25 ml of the sensitized cell suspension to each tube in the diagnostic test, including the complement control tubes.

10. Incubate the tubes for 30 minutes at 37°C in the water bath to complete the test.
11. Before the tests are read, 1 ml of cold diluent is added to each complement control tube, the tubes are placed in the centrifuge and the cells deposited, after which the supernatants are amalgamated and the percentage haemolysis is determined. For the test results to be valid the percentage haemolysis should be between 25% and 60%. If a photometer is not available, the percentage haemolysis can be determined by using haemolysis standards.

12. In reading the tests the endpoints are normally sharp and can be assessed directly. Complete fixation (no haemolysis) is recorded as ++ +++, complete lack of fixation (complete haemolysis) is recorded as 0. Degrees of partial haemolysis are awarded −−−−, ++ (50% haemolysis) or +. If desired, the percentage of haemolysis present in tubes showing less than complete lysis may be determined exactly by reference to colour standards or by use of a photometer.

The micro technique

The microtest is carried out in plastic trays with U-shaped rather than V-shaped wells, of a size suitable for working with volumes of 100 μl. The Microtiter system is suitable. The volumes of reagents used are one-tenth of those used in the macrosystem, described above. Haemolysin, complement, and antigen are more conveniently titrated in 1-ml volumes in tubes, as already described; the same titres are, however, obtained if the titrations are done in microvolumes.

The serum is conveniently dispensed with a sampler having a disposable tip (one type of which is shown in Fig. 20) and may be diluted with the

FIG. 20. SAMPLER WITH DISPOSABLE TIPS

1 Supplied by: Cooke Engineering Co., Alexandria, Va., USA.
hand operated mechanical diluter (shown in Fig. 21) which is capable of diluting up to 12 sera simultaneously. These instruments should be used strictly according to the manufacturers' instructions.

FIG. 21. MICROTITER DILUTOR

To prepare for the serial dilution of the serum under test, 25 μl of diluent is added with a dropper to each well in the plastic plate except the first of each row. 25 μl of the inactivated serum dilution of the first test sample is placed in the first, second, and last well of the first row. 25-μl amounts of the second test sample are similarly dispensed in the second row and so on. Serial dilutions are made in all rows simultaneously with the mechanical diluter, starting with the second well and finishing with the last well but one, from which the mechanical diluter removed 25 μl.

After the sera have been diluted, 25 μl of antigen appropriately diluted is dropped into each well, except the last in each row which serves as an anti-complementary serum control, followed by 25 μl of complement in all wells. The reagents are mixed by tapping gently on the side of each plate. A reference serum should be included with each set of tests and a control on the activity of the complement is carried out in tubes as described on pages 100-101, paragraphs 7 and 11. The plates are covered and placed

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1 Supplied by: Cooke Engineering Co., Alexandria, Va., USA.
in an incubator at 37° C for half an hour, or in the refrigerator at 0-4° C overnight (14-18 hours). After the period of fixation is completed, 25 μl of sensitized erythrocytes are added to each well and the plate covered with transparent adhesive tape. It is important that the sensitized erythrocytes be thoroughly mixed with the other components of the test and kept in suspension. The final incubation is in the incubator at 37° C for half an hour and further agitation of the mixtures after 15 minutes incubation is advisable. A mechanical shaker designed to hold the trays and fitted inside the incubator is recommended to provide agitation of the plates throughout the 30-minute incubation period.

The results are read after the plates have been centrifuged in the Microtiter carrier plate, or after standing for 2–3 hours in the refrigerator. The plate is best examined from underneath, preferably with the help of a magnifying mirror. In the absence of haemolysis a button of erythrocytes is seen at the bottom of the well and the supernatant is clear and colourless (a ++++ reaction); with complete lysis there is no button of erythrocytes and the liquid in the well is clear and coloured with haemoglobin (a negative reaction). Various degrees of incomplete lysis are noted as +++, ++, and + + reactions.

Haemolysis standards, representing 0, 25, 50, 75, and 100% haemolysis prepared in the manner described in Table 10, page 95, may be placed in empty wells of the plate at the time the sensitized cells are added (four 25-μl drops per well).

A selection of mechanical devices is available\(^1\) for adding reagents in the micro test and for making doubling dilutions of serum with up to 12 sera being diluted simultaneously. When large numbers of sera are being tested much tedious can be avoided by the use of mechanical methods and personnel remain alert to supervise the working of the system.

The one-tube screen test

The occurrence of prozones with warm fixation means that cold fixation is obligatory for the one-tube screen test. In the screen test, serum controls are not required as positive sera will be retested (with anti-complementary controls) and negative sera cannot be anti-complementary at the dilution tested.

Tubes, rather than plastic plates, are used; 0.025 ml of the serum sample is placed in the tube by means of a sampler which has a disposable tip and 0.225 ml of diluent is added with an automatic pipette. These serum dilutions are inactivated by placing the tubes in a water bath at 62° C for 30 minutes and subsequently stored in the refrigerator. At the end of the day's work, 0.25 ml of antigen is dispensed automatically into each tube, followed by 0.25 ml of complement, and the tubes are returned to the refrigerator.

\(^1\) Suppliers: Caninco, Fisher Lane, Rockville, Maryland, USA; Cooke Engineering Co., Alexandria, Va., USA.
Alternatively, equal quantities of antigen and complement may be mixed and 0.5 ml dispensed into each tube. Next morning (after a fixation period of 14–18 hours) 0.25 ml of sensitized cells is dispensed automatically into each tube, the tubes are held for 30 minutes in the water bath at 37°C, and then the results are read. A standard serum diluted past its known endpoint is included with each day's tests and complement controls are set up in the same way as for the diagnostic test (see pages 100-101, paragraphs 7 and 11). Sera positive to the one-tube test are retested in serial dilutions as in the diagnostic test and the results are interpreted accordingly.

**Anti-complementary sera**

The following method is often successful for retesting anti-complementary sera:

(a) Make a 5% solution of bovine serum albumin (BSA) fraction V (Armour list 2293), in CF diluent.

(b) Prepare the first dilution to be used in the test in this albumin solution (say 0.2 ml of serum and 0.8 ml of BSA solution).

(c) Incubate in the water bath at 37°C for 30–60 minutes.

(d) Inactivate at the temperature appropriate to the type of test being used and proceed with the test as usual.

**Interpretation of complement fixation titres**

The endpoint of the reaction is taken as the last dilution showing appreciable fixation (traces are ignored). This dilution is taken as the titre of the serum. The macro system as described produces a titre of 1:640 on cold fixation and 1:256 on warm fixation with the second International Standard Anti-Brucella abortus Serum (ISAbS). When testing the sera of cattle, sheep, and goats it is recommended that titres equal to or greater than 1:20 for cold fixation and 1:8 for warm fixation be taken to indicate the presence of infection. The Microtiter system has been found to give a titre about 1 tube less than the macro system and therefore 1:10 for cold and 1:4 for warm fixation are the titres to be considered as positive.

It is also recommended that complement fixation titres should be expressed in relation to the titre given by the second International Standard Anti-Brucella abortus Serum with the method in question.

**Hill's complement fixation method for bovine brucellosis**

The method to be described is a modified form of that described by Hill (1963). The test is conducted in clear plastic plates with wells that will

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* A 5% solution of guinea pig complement may be used instead.
hold at least 1 ml of reagent. The WHO-type haemagglutination plates are suitable. Perspex plates with flat-bottomed wells 13 mm in diameter and 16 mm deep are also suitable; 2 sizes of these plates are convenient, one 14 × 19 cm with 96 wells and the other 19 × 19 cm with 144 wells.

The test uses warm fixation (37° C). The water bath should contain sufficient water to cover the bottom and to come approximately 5 mm up the sides of the plate. Where the same quantity of any reagent needs to be dispensed into a number of wells, this may conveniently be done with an automatic pipette.

After use, the plates should be thoroughly rinsed with tapwater, then rinsed 3 times with distilled water and placed in a 37° C incubator to dry. If necessary, the plates can be cleaned with dilute chromic acid cleaning fluid (see page 87).

**Reagents**

*Stock barbital-buffered saline solution*

Barbital-buffered saline solution is used in preparing all reagents. A fivefold concentrated stock solution is prepared as described on page 88; it may be stored at 4° C for an extended period of time.

*Buffer working solution*

To prepare a working dilution of buffer, 1 part of the stock solution is diluted with 4 parts of distilled water containing 400 mg of Bacto-gelatin per litre.

*Sheep erythrocytes*

Sheep erythrocytes are collected and stored in Alsever’s solution as described on page 89. A 3% erythrocyte suspension is prepared for the test using one of the methods described on pages 91–92.

*Haemolysin*

Haemolysin is prepared as described on page 90; its titration is described later.

*Complement*

The production and storage of complement have been discussed on page 90. Titration of complement is described later.

*Antigen*

The USDA tube agglutination test antigen, produced as described on pages 70–72, is used for the test. Alternatively, the European agglutination test antigen may be used (see pages 79–80).
Standardization of reagents

**Haemolysin**

(a) Prepare twofold dilutions of haemolysin (1:100–1:12,800).

(b) Mix each dilution of haemolysin with an equal volume of the standardized erythrocyte suspension. Allow to stand for 15 minutes.

(c) Make a 1:30 dilution of complement.

(d) Identify wells on the plastic plate to correspond with each dilution of haemolysin. Include an extra well as the sensitized erythrocyte control.

(e) Add 0.4 ml of buffer to each haemolysin well and 0.6 ml to the erythrocyte control well (Table 14).

### TABLE 14: EXAMPLE OF HAEMOLYSIN TITRATION

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Haemolysin dilution</th>
<th>Erythrocyte control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:100</td>
<td>1:200</td>
</tr>
<tr>
<td>Buffer (ml)</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Complement (ml)</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Sensitized erythrocytes (ml)</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Incubate at 37°C for 30 minutes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Results</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*(a) One unit of haemolysin is the highest dilution that produces 100% haemolysis (1:1600 in the table above). Five units of haemolysin are used in the test.*

(f) Add 0.2 ml of complement to all wells except the erythrocyte control well.

(g) Add 0.4 ml of each of the various haemolysin-erythrocyte mixtures to the corresponding wells (the erythrocyte control well receives 0.4 ml of erythrocytes sensitized with the 1:100 dilution of haemolysin).

(h) Mix the contents by rotating the plate, place it in a water bath at 37°C for 30 minutes and read.

**Titration of complement**

1. Prepare a dilution of haemolysin containing 5 haemolytic units (see above).

2. Prepare a 3% suspension of sheep erythrocytes.

3. Prepare a 1:10 master dilution of complement in diluent at 0–4°C and from this prepare further dilutions as shown in Table 15.
### TABLE 15. TITRATION OF COMPLEMENT

<table>
<thead>
<tr>
<th>Dilutions of complement</th>
<th>1:30</th>
<th>1:40</th>
<th>1:50</th>
<th>1:60</th>
<th>1:70</th>
<th>1:80</th>
<th>1:90</th>
<th>1:100</th>
<th>1:110</th>
<th>1:120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complement (ml)</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Diluent (ml)</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Sensitized erythrocytes (ml)</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Incubate at 37°C for 30 minutes

Reincubate at 37°C for 30 minutes

4. Using 10 suitable tubes or wells in a plastic plate, 0.4 ml of diluent is placed in each, followed by 0.2 ml of the complement dilutions as shown in Table 15.

5. Place the dilutions in a water-bath at 37°C for 30 minutes.

6. Mix 1 volume of the haemolysin dilution prepared in Step 1 above with 1 volume of the 3% sheep erythrocyte suspension prepared in Step 2. After 15 minutes at room temperature this makes a sensitized erythrocyte suspension.

7. At the completion of the 30-min incubation, add 0.4 ml of sensitized erythrocytes to each tube or well.

8. Reincubate at 37°C for 30 minutes.

9. The titre of the complement is determined as the last dilution to give complete haemolysis.

10. 1½ units of complement are used in the diagnostic test; this dilution is calculated as follows: suppose 1 unit = 1/50 then 1½ units = 1/50 × 5/4 = 1/40.

### Titration of antigen

If the USDA tube agglutination test antigen (or equivalent) is used it may be used diluted 1:200 in buffer without being titrated. Similarly the European agglutination test antigen may be used diluted 1:200, i.e., at 1:20 of the dilution used for the agglutination test.

If it is desired to titrate the antigen this may be done in a block titration as illustrated in Table 16. A moderately high titre serum is diluted 1:4 and inactivated at 58°C for 50 minutes. Further twofold dilutions of the inactivated serum are made and dispensed in the vertical rows of wells as shown in Table 16, 0.2 ml per well; the wells in the last row receive 0.2 ml diluent each in lieu of serum. Twofold dilutions of antigen (agglutinating concentrate) are made as shown in the table and dispensed in the horizontal rows including the anti-complementary controls (0.2 ml per well), and 0.2 ml of
complement containing $1\frac{3}{4}$ units (see above) is added to all wells. Another well is required as anti-complementary control for the serum; it contains 0.2 ml of a 1:4 dilution of the inactivated serum, 0.2 ml of diluent in lieu of antigen, and 0.2 ml of complement. A set of complement controls as described under the diagnostic test (control (c) on page 109) are included.

The plate is placed in the water bath at 37° C for 30 minutes after which 0.4 ml of sensitized erythrocytes is added to all wells and the plate reincubated for 30 minutes before the test is read.

There should be no anti-complementary activity in either the antigen or serum controls as evidenced by the occurrence of complete haemolysis in these wells. The most sensitive dilution of antigen (1:200 in the example shown in Table 16) is normally selected for use in the test; however, the most concentrated antigen suspension compatible with a high level of sensitivity should be used to limit the occurrence of prozones in the diagnostic test and some workers would prefer to select the 1:100 dilution in the example given in Table 16.

The diagnostic test

1. Dilute sera to be tested with equal quantities of diluent (say 0.4 ml of serum and 0.4 ml of diluent).

2. Inactivate at 58° C for 50 minutes.

3. Five wells in the plastic plate (tubes may be used if desired) are required for each serum being tested; the fifth forms a control on the anti-complementary activity of each serum. Take up 0.56 ml of the inactivated 1:2 dilution of serum in a pipette or 1-ml tuberculin syringe (see page 82) and add 0.2 ml, 0.1 ml, 0.04 ml, 0.02 ml, and 0.2 ml to the first, second, third, fourth, and fifth wells, respectively, to make serum dilutions of 1:2, 1:4, 1:10, 1:20 and a 1:2 serum anti-complementary control.

4. Add 0.1 ml of diluent to the second well, 0.2 ml to the fifth, and 0.17 ml to the third and fourth wells.
5. Add 0.2 ml of antigen to each of the first four wells.
6. Add 0.2 ml of complement to each well.
7. Incubate plates in a 37° C water bath for 30 minutes.
8. Mix together equal volumes of 3% sheep erythrocytes and haemolytic serum containing 5 units per ml and allow to stand for 15 minutes.
9. Immediately after the incubation of the plates is completed add 0.4 ml of the mixture to each well.
10. Incubate the plates in a 37° C water bath for a further 30 minutes.
11. The reactions are best read with the plate held over a light source. Express the degree of fixation as 0 (complete haemolysis, liquid in well completely clear) +, ++, ++++, and ++++ according to the proportion of erythrocytes remaining unlysed (different degrees of cloudiness).
12. The titre of the serum being tested is expressed by writing the degree of fixation in the last well showing a reaction (as represented by the number of + signs) over the dilution of serum in this well, e.g., a ++ reaction at a dilution of 1:2 is expressed as 2/2.

**Controls**

(a) A control on the anti-complementary activity is done for each serum and has already been described. Sera showing anti-complementary activity should be subjected to another type of test; it is recommended that the result of the BBA tests (see page 110) be accepted.

(b) A positive serum with a titre within the range of dilutions being tested and a negative serum are included with each set of tests.

(c) A simplified complement titration is included along with each set of tests. Four wells are used as follows:

<table>
<thead>
<tr>
<th>Diluent (ml)</th>
<th>0.4</th>
<th>0.45</th>
<th>0.5</th>
<th>0.55</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complement (ml)</td>
<td>0.2</td>
<td>0.15</td>
<td>0.1</td>
<td>0.05</td>
</tr>
</tbody>
</table>

The plate is incubated and the sensitized erythrocyte suspension added in the same way as in the diagnostic tests. The results in the four wells should be 0, trace, trace, and ++ +, respectively.

**Interpretation of results**

In Britain, for the diagnosis of bovine brucellosis, the complement fixation test is carried out in the manner described above using antigen standardized to give 50% fixation at a dilution of 1:220 of the second International Standard Anti-Brucella abortus Serum. The results are interpreted as shown in Table 17.
TABLE 17. INTERPRETATION OF THE COMPLEMENT FIXATION TEST—a
(HILL’S METHOD)

<table>
<thead>
<tr>
<th>Animal</th>
<th>Pass</th>
<th>Inconclusive</th>
<th>Fail</th>
</tr>
</thead>
<tbody>
<tr>
<td>Officially vaccinated (strain 19)</td>
<td>Negative to 1/4</td>
<td>2/4 to 1/10 (fail at 3rd test)</td>
<td>2/10 and over</td>
</tr>
<tr>
<td>Nonvaccinated, or of unknown vaccination status, and bulls</td>
<td>Negative to 1/2</td>
<td>2/2 to 1/4 (fail at 3rd test)</td>
<td>2/4 and over</td>
</tr>
</tbody>
</table>

—a The denominator is the highest dilution showing fixation and the numerator the degree of fixation at this dilution according to the number of plus signs recorded.

Automation of the complement fixation test

Mechanical aids for complement fixation test manipulations have already been mentioned (page 103). A continuous flow system is also available (Elliott & Pullan, 1973). This system is in routine use at the Central Brucellosis Laboratory in New Zealand and the Officer-in-charge is prepared to supply details to recognized laboratories.

The system used in New Zealand is completely automated; the serum sample is taken up and the various reagents are added automatically, mixing of succeeding samples being prevented by the regular introduction of air bubbles. The degree of haemolysis occurring in each sample is measured in a colorimeter and shown as a tracing on a recorder.

The buffered Brucella antigen tests

The buffered Brucella antigen tests (card test and Rose Bengal plate test) are rapid agglutination tests that employ a Rose Bengal stained antigen buffered at pH 3.65 and adjusted to contain 8% of cells by volume, as determined by the packed cell volume method.

Preparation of the buffered Brucella antigen (BBA)

Buffered diluent

Weigh out 120 g of sodium hydroxide, add 2 litres of sterile 0.85% sodium chloride solution containing 0.5% phenol and mix. Add 540 ml of concentrated lactic acid and mix. Add sufficient sterile 0.85% sodium chloride solution containing 0.5% phenol to make 6 litres.

Rose Bengal stain

Weigh out 4 g of Rose Bengal, add 386 ml of sterile distilled water, and mix.

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1 Supplied by Technicon Instruments Corporation, Tarrytown, N.Y., USA.
2 Central Brucellosis Laboratory, Upper Hutt, New Zealand.
Staining the brucellae

Prepare a dense suspension of *B. abortus* strain 1119-3 according to the procedures already described on pages 65–70. The preparation of a batch of approximately 6 litres is described below; some laboratories may prefer to prepare larger or smaller batches. Pour a quantity of suspension containing approximately 550 g, packed wet cell weight, of *B. abortus* strain 1119-3 into a sterile bottle of approximately 20 litres capacity and add sufficient sterile 0.85% sodium chloride solution containing 0.5% phenol to bring the volume to 22.5 ml per gram of cells. Add 1 ml of Rose Bengal solution per 35 ml of this suspension and then mix with a magnetic stirrer for 2 hours. Filter the mixture through sterile absorbent cotton, remove the stained brucellae by centrifugation in a refrigerated centrifuge, and determine and record the weight of *Brucella* cells. Add approximately 2 ml of buffered diluent per gram of stained brucellae, and suspend the brucellae by mixing on a shaking machine for 2 hours.

Standardization

Transfer the stained suspension to a sterile 9-litre bottle and add buffered diluent to bring the volume to 14 ml per gram of stained brucellae as previously recorded. Add a Teflon-coated magnetic bar, stir for 2 hours, and filter through absorbent cotton. The suspension is again stirred for 2 hours in the same way and a quantity is removed for determining the density by the packed-cell volume method as described on page 61. In determining the density, 0.5 ml of the suspension is measured into each Hopkins tube. Four control tubes each containing 0.5 ml of a suspension known to contain 8% of *B. abortus* 1119-3 cells should be centrifuged at the same time. If the volume of cells in the suspension under test does not equal 8%, the concentration is adjusted by adding buffered diluent or stained brucellae, stirring for 2 hours and redetermining the density until the proper density is achieved. Determine the pH, which should be 3.65±0.05. Mix equal quantities of bovine serum and antigen and again determine the pH, which should now be 3.80±0.05 and comparable to previous lots. Conduct sensitivity tests on at least 10 serum samples and compare the results with 1 or more previous lots of antigen. The antigen is mixed overnight on a magnetic stirrer and dispensed into bottles or vials. The bottles or vials are labelled and the expiry date (1 year) is added. The antigen is stored at 4°C until used.

Procedures for the buffered *Brucella* antigen tests

*Card test (USA)*

All components for the card test are disposable and are provided as field or laboratory kits.\(^1\) The field kits provide plasma collection equipment

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\(^1\) Supplied by Hyson, Westcott & Dunning, Inc., Baltimore, Md., USA.
that allows rapid separation of plasma. The laboratory kits are for testing serum in the laboratory. Equal quantities (0.03 ml) of serum or plasma and buffered antigen are placed on a tear-drop test area on the card, mixed and spread over the tear-drop area, and the test is read immediately after a 4-minute rocking period.

*Rose Bengal plate test (Great Britain)*

Buffered *Brucella* antigen prepared and standardized as described on pages 110 and 111 is used in Great Britain in a laboratory test. Equal quantities of serum and antigen (0.03 ml) are measured on white enamel or plastic trays, mixed, and read immediately after a 4-minute rocking period.

**Interpretation of results**

The buffered *Brucella* antigen tests are read as positive (any degree of agglutination) or negative (no agglutination). See introduction on page 64 for further discussion on the interpretation of results.

**The mercaptoethanol test**

This test is an agglutination test carried out in the presence of 2-mercaptoethanol, which inactivates IgM molecules present in the serum being tested; thus the test may be looked upon as an indicator of the amount, if any, of anti-*Brucella* IgG agglutinin present in the serum. Both the USDA and the European tube agglutination tests described on pages 70 and 79 may be easily modified to become mercaptoethanol tests.

A 0.1 mol/litre mercaptoethanol solution in normal saline is made up as follows:

- sodium chloride, 8.5 g
- 2-mercaptoethanol, 7.14 ml
- distilled water, to 1 litre

The solution should be stored at 4°C and prepared fresh every 2-3 weeks. Phenol should not be used at any stage in the mercaptoethanol test. Whichever technique is used, the tubes are incubated and the results read and recorded in the same way as with the corresponding tube agglutination test.

**The USDA tube mercaptoethanol test**

Quantities of 0.08 ml, 0.04 ml, 0.02 ml, and 0.01 ml of the serum under test are placed in 4 tubes as described on page 72 (point 3) and 1 ml of 0.1 mol/litre mercaptoethanol in saline and 1 ml of concentrated USDA tube antigen diluted 1:50 in normal saline solution are added to each tube.
The final concentration of mercaptoethanol is 0.05 mol/litre. The tubes are then shaken and incubated as in the tube agglutination test.

**The European tube mercaptoethanol test**

The serum dilutions are made in mercaptoethanol saline and the antigen concentrate is diluted in normal saline solution without phenol; otherwise the technique is the same as for the European tube agglutination test as described on page 79.

**Interpretation of the results**

It is advisable to interpret the titres obtained in the mercaptoethanol test alongside those for the ordinary tube agglutination test. It may be assumed that the ordinary agglutination titre indicates the total amount of agglutinins present, the mercaptoethanol test titre the amount of IgG agglutinin, and the difference between these two titres the proportion of IgM agglutinin present in terms of agglutinating activity.

IgG is generally associated with the presence of active infection and any positive titre in the mercaptoethanol tests just described should be regarded as indicating infection, or at least suspicion of infection. IgM agglutinins occur in animals with recent acute infections, generally in high titre, but low titres in uninfected animals vaccinated more than 1 year previously are often also caused by IgM agglutinins. Thus in cattle, sheep, and goats moderate to low static agglutination titres in animals vaccinated some time previously with live vaccines are considered to have no significance when the mercaptoethanol test is negative, but to indicate the presence of *Brucella* infection when the mercaptoethanol test is positive.

**The Coombs (antiglobulin) test**

Certain sera contain specific antibodies that combine with antigen but are not capable of causing agglutination; some of these antibodies, presumably by occupying combining sites on the antigen, prevent agglutinins from causing agglutination. The Coombs test utilizes the Coombs reagent to bring about agglutination in the presence of these so-called incomplete antibodies. The Coombs reagent is an antiserum specific against either globulin or whole serum of the species of animal whose serum is being tested. The Coombs test is sometimes known as the anti-human globulin (AHG) test when applied to human sera, and the anti-bovine globulin (ABG) test when applied to bovine sera.

Any ordinary tube agglutination technique may be used as a starting point for the Coombs test; the tubes that do not show agglutination are retested in the presence of the Coombs reagent after the antigen with
adherent antibody, if any is present, has been washed by repeated centrifugation and resuspension. A Coombs test technique based on the European agglutination test is given here. A different technique developed by Hajdu (1958) has been used successfully in Czechoslovakia in the diagnosis of bovine brucellosis.

Technique of the "European" test

The ordinary tube agglutination test is carried out as described on page 81, except that it is preferable to use the antigen suspended in normal saline rather than phenol-saline. The agglutinating antigen concentrate is centrifuged twice at 10,000 g for 20 minutes and resuspended each time in normal saline, the second time to 10× the original volume of concentrate used. This produces an antigen of the correct turbidity. After the result of the agglutination test has been read, the negative tubes are identified individually and centrifuged at 2500 g for 20 minutes and the supernatant is poured off; care should be taken to avoid heating the tubes during centrifuging as this may cause elution of the antibody. The deposit is resuspended to the original volume in normal saline; the saline may be added with an automatic pipette or a rheometer syringe, after which the deposit is completely resuspended by the use of a Pasteur pipette fitted with a rubber bulb, starting with the tube that had the highest serum dilution and working towards the tube that had the lowest. A clean Pasteur pipette is required for each set of tubes. Alternatively the deposit may be resuspended after pouring off the supernatant fluid, by agitating the tubes on a lateral shaker for 10 minutes to resuspend the deposit in the drop of supernatant fluid remaining in the tube, and then adding the required volume of normal saline from an automatic pipette; hand shaking now suffices. The washing process is repeated 3 times; after the third centrifuging the deposit is resuspended in the Coombs reagent diluted in normal saline as described below under titration of the Coombs reagent. The tubes are replaced in the incubator for approximately 24 hours, after which they are read in the same way as in the ordinary agglutination test. The titres normally given indicate the highest dilution in which 50% or more agglutination occurs, irrespective of the result given by the ordinary tube agglutination test.

A modification of the above procedure, in which the final stage is changed to a spot test, is preferred by some workers. After the tubes have been centrifuged for the third time the deposits are each resuspended in 3 or 4 drops of normal saline and one drop of each transferred to a marked slide or glass plate; this results in a row of drops corresponding to the number of serum dilutions being tested. One drop of appropriately titrated and diluted Coombs reagent is added to each drop and mixed with a toothpick. The slides are incubated at 37°C for 30 minutes in a closed humid container after which the mixtures are examined with a low-power microscope or
a hand lens for visible agglutination. Some workers prefer to duplicate the drops of resuspended deposit on the slide, to test one row with the Coombs reagent and the other row with normal saline. This indicates whether the reaction is caused by the Coombs reagent or has resulted simply from washing and resuspending the antigen-antibody complexes.

**Interpretation of the results**

Using the technique described above, a titre of 1:40 must be taken to indicate that the patient has suffered a specific *Brucella* stimulus. The test has found practical application in human medicine, especially in the diagnosis of chronic brucellosis, where a positive Coombs test titre may be present when the ordinary agglutination titre is either low or absent altogether. The Coombs test is, however, extremely sensitive and individuals exposed to *Brucella* infections, such as veterinarians and laboratory workers, often have high Coombs test titres without any symptoms of illness. On the other hand, a continuous negative reaction to the Coombs test in a sick person is very strong evidence against brucellosis being the cause of the illness.

**Preparation of the Coombs reagent**

Species-specific Coombs reagent, e.g., anti-human globulin and anti-ovine globulin, can be purchased. Coombs reagent against these and other species may be prepared in the laboratory, the technique being the same whatever species the reagent is being prepared against. The rabbit is a suitable animal for this purpose, except, of course, in the unlikely event of anti-rabbit globulin being required. There are a number of different techniques for preparing these antisera; of the two given here, one is for producing anti-globulin serum and the other for producing anti-whole serum.

In the first technique globulin is separated from whole serum by precipitation with alum according to the method of Proom (1943).

1. Dilute 25 ml of serum, pooled by mixing samples from several animals of the species required, with 80 ml of distilled water.

2. Add 90 ml of a 10% aqueous solution of potassium aluminium sulfate (KAl(SO₄)₂·12H₂O).

3. Adjust the pH of the mixture to 6.5 by adding a 5 mol/litre sodium hydroxide solution drop by drop.

4. Centrifuge the mixture and remove the supernatant fluid.

5. Wash the precipitate twice with 200 ml of a 1:10 000 solution of thiomersal in normal saline and finally make the washed precipitate up to a volume of 100 ml with this solution.
The suspension can be stored for at least 14 days at 0–4°C. For injection it may conveniently be emulsified in adjuvant. For example:

50 parts of globulin suspension
4 parts of Arlacel A
50 parts of light mineral oil

Or the globulin suspension may be emulsified in an equal volume of commercial Freund’s incomplete adjuvant.

Inject 1 ml of this emulsion into the upper, muscular area of each hind leg of the rabbit. The injections are repeated 14 days later, and after another 10–14 days have elapsed the animal is bled.

When whole serum is used for producing the Coombs reagent, 0.5–1 ml of undiluted serum is injected intraperitoneally. This is followed within 24 hours by intravenous injection into the ear vein of 0.5 ml of serum diluted with an equal volume of normal saline. For the next 3–4 days daily injections of 1 ml of serum are given either intravenously or intramuscularly. The rabbits are bled approximately 10 days after the last injection.

The Coombs reagent prepared by either of the methods described above may be stored at —20°C or lower, or, preferably freeze-dried.

Titration of the Coombs reagent

To titrate the Coombs reagent, a Coombs test positive serum of the species of animal in question is required, i.e., a serum that has a Coombs test titre of at least 4 times its titre in the routine agglutination test; ideally a serum that is negative to the ordinary agglutination test and highly positive to the Coombs test should be used.

1. An ordinary tube agglutination test is carried out on the known Coombs test positive serum. This is done in at least 5 rows of identical doubling dilutions with sufficient tubes in each row to carry the dilutions beyond the Coombs test titre of the serum.

2. After the appropriate period of incubation, any tubes showing complete agglutination are discarded and the remainder are used for a block titration of the Coombs reagent as shown in the example given in Table 18.

3. Before the Coombs reagent can be added, the tubes must be centrifuged, the supernatent fluids discarded, and the deposit resuspended to its original volume as in the test proper; this process is repeated 3 times, but after the third centrifuging the deposit is resuspended in the Coombs reagent diluted with normal saline, a different dilution for each horizontal row of tubes as shown in Table 18.

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1 Either Bayol F supplied by the Exxon Petroleum Company or Ondina 17 supplied by the The Shell Petroleum Company, or an equivalent oil.
TABLE 18. TITRATION OF COOMBS REAGENT

<table>
<thead>
<tr>
<th>Coombs reagent</th>
<th>Serum dilutions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:10</td>
</tr>
<tr>
<td>1:50</td>
<td>++++</td>
</tr>
<tr>
<td>1:100</td>
<td>++++</td>
</tr>
<tr>
<td>1:200</td>
<td>++++</td>
</tr>
<tr>
<td>1:400</td>
<td>++++</td>
</tr>
<tr>
<td>1:800</td>
<td>++++</td>
</tr>
</tbody>
</table>

4. After a further period of approximately 24 hours in the incubator at 37°C the result is read and recorded in exactly the same way as in the ordinary agglutination test.

If the Coombs reagent is being titrated for use in the modified technique ending in a slide test, then in the final stage of the titration the dilutions of the Coombs reagent must be added to the reconstituted deposits on slides and treated as previously described for the slide test (page 114).

The dilution of the Coombs reagent to be used in the diagnostic test is the most dilute solution to show maximum sensitivity in the titration. In practice, it may be preferable to use a slightly less dilute suspension than that defined above, e.g., with the Coombs reagent whose titration is shown in Table 18, a dilution of 1:100 would be suitable for use in the diagnostic test.

The Brucella milk ring test

The Brucella ring test (BRT) is a screen test to determine the possible presence of brucellosis in a herd and is the most practical method for locating infected dairy herds. Herds from which the milk ring test is positive should be examined by serological tests to identify the infected animals. In many areas milk is now collected in bulk tankers and dilution factors are greatly increased as compared with milk transported in cans. The implications of these changes for the BRT have been examined by Roepke & Stiles (1970), who conclude that the BRT is still a valuable test when used on bulk samples, provided the size of the herd being sampled is taken into consideration in evaluating the results.

Two types of antigen are used in the test: the blue haematoxylin-stained antigen and the red tetrazolium-stained antigen. Except for the difference in colour, the characteristics of these two antigens are very similar. The haematoxylin-stained antigen is generally used for testing cow's milk, while some workers prefer the tetrazolium-stained antigen for testing the milk of sheep and goats.
Haematoxylin antigen

The following is the method of preparation employed by the Veterinary Services Laboratories, US Department of Agriculture, Ames, Iowa. The quantities of reagents given here are one-tenth of those used by the USDA.

Preparation of the staining solution

1. Dissolve 9 g of ammonium aluminium sulfate \((\text{NH}_4\text{Al(SO}_4)_2\cdot12\text{H}_2\text{O})\) in 100 ml of distilled water by heating to 90° C. Add 30 ml of glycerol.

2. Dissolve 1.9 g of haematoxylin\(^1\) in 10 ml of ethanol (95%) by heating to 50-60° C. Make up to 100 ml by adding distilled water.

3. Dissolve 0.2 g of sodium iodate in 2 ml of distilled water by heating to 90° C.

4. Mix the reagents prepared in steps (1) and (2), and to this mixture add the reagent prepared in step (3). The solution is mixed thoroughly by shaking and is allowed to stand for 30 minutes at room temperature to permit oxidation to take place. The oxidation reaction can then be observed by a series of colour changes, the final colour being a dark purple with a metallic sheen.

5. A 10% solution of ammonium aluminium sulfate is prepared by dissolving 94 g of ammonium aluminium sulfate in 500 ml of distilled water by heating to 90° C. Distilled water at room temperature is added to bring the volume to 940 ml.

6. The staining solution described in step (4) is then diluted with the 940 ml of ammonium aluminium sulfate solution.

7. After cooling to room temperature, the pH of the staining solution is determined and, if necessary, is adjusted to pH 3.1 by adding sufficient 10% sodium hydroxide solution.

8. The staining solution is stored at room temperature in the dark for 45-90 days.

9. Before use, the staining solution is shaken and then filtered through an absorbent cotton filter to remove the insoluble substances and precipitates.

Staining the antigen

1. Heat-killed Brucella organisms, prepared as described on pages 65-70, are added at the rate of 45 g of packed cells per 1000 ml of the staining solution. The suspension is held at room temperature under constant agitation by a stirring apparatus for 48 hours. During this period the cells will be uniformly stained a bluish purple.

\(^1\) Obtainable from the Fisher Scientific Co., Fair Lawn, N.J., USA, or other suppliers of certified reagents.
2. A solution for washing the stained cells is prepared by adding 6.4 g of sodium chloride, 1.5 ml of lactic acid (85% grade), and 4.4 ml of 10% sodium hydroxide solution to 1600 ml of distilled water. The pH should be determined and, if necessary, adjusted to 4.0 by adding either lactic acid or a 10% solution of sodium hydroxide.

3. The stained Brucella cells are centrifuged, the supernatant is discarded and the cells are resuspended in the washing solution described above to make a final volume approximately 3 times their packed-cell volume. The cells must be thoroughly resuspended by mechanical shaking or stirring. More washing solution is added to the resuspended cells to bring the volume to 30 times the packed-cell volume. This suspension is mixed thoroughly and centrifuged again; the supernatant is discarded and the packed cells are again resuspended in the same quantity of washing solution. They are centrifuged a third time and the supernatant is discarded.

4. The final diluent for suspending the stained cells is 0.85% sodium chloride solution containing 0.5% phenol, which has been adjusted to pH 4.0 by the addition of 0.1 mol/litre citric acid solution and 0.5 mol/litre disodium hydrogen phosphate solution. (About 2.6 ml of 0.1 mol/litre citric acid solution and 1 ml of 0.5 mol/litre disodium hydrogen phosphate solution per 1000 ml of phenol saline should result in pH 4.0.)

5. The stained Brucella cells are suspended in the above diluent, at the rate of 1 g of packed cells to 27 ml of diluent, and mixed thoroughly. The mixture is then placed in a refrigerator for 24 hours, after which time it is mixed again, passed through a cotton filter, and the pH determined. The final pH should be between 4.0 and 4.3. (It can be adjusted by the addition of the citric acid or disodium hydrogen phosphate solutions described above.)

6. The packed-cell volume of the final product is determined by adding 1 ml of the antigen to 4 ml of distilled water in each of 4 Hopkins centrifuge tubes. The total cell concentration is determined as described on page 61. If the packed-cell volume does not equal 4%, the concentration is adjusted by adding either stained Brucella cells or the final diluent.

The sensitivity of each new lot of antigen is compared with a standard lot of antigen in the ring test. These tests are conducted on a group of 5 or more high-titre milk samples serially diluted in untreated negative milk from a brucellosis-free herd containing at least 25 lactating cows. A standard lot of antigen is prepared each year and each new lot of antigen is compared in sensitivity to this standard. This minimizes the danger of slight cumulative errors that might occur from comparing successive lots.

For those laboratories that prepare only one batch of antigen per year it would be advisable to standardize it against a national standard serum, as described below for the standardization of the tetrazolium antigen.
Tetrazolium ring test antigen

Preparation

The bacterial suspension is prepared as described on pages 65-70. Normal saline is used in place of phenol-saline to harvest a live suspension of bacteria. Add 1 g of 2,3,5-triphenyl-tetrazolium, or 0.8 g of 3,5-diphenyl-tetrazolium per 500 ml of freshly harvested live bacterial suspension. The mixture is shaken vigorously and continuously for 10 minutes and kept for 2 hours at 37° C and then for 30 minutes at 65° C in a water bath. The suspension will now be of a deep cherry-red colour. The cells are deposited by centrifugation and the supernatant liquid, which is very faint yellow in colour, is discarded. The sediment is resuspended in water containing 0.85% of sodium chloride, 1% of glycerol, and 1% of phenol and the suspension is filtered through a very thin layer of cotton wool and gauze, all clumps having previously been dispersed.

Standardization

The stained antigen is standardized against either the International Standard Anti-Brucella abortus Serum (ISAbS) or a national standard with the same titre. A portion of the bacterial suspension stained with tetrazolium is taken, representing the initial undiluted suspension. To each of 6 test tubes, 1 ml of this initial undiluted stained suspension is added, followed by increasing quantities of the glycerol-phenol diluent as follows:

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Undiluted stained suspension (ml)</th>
<th>Diluent (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>0.6</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>0.8</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>1.0</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>1.2</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>1.4</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>1.6</td>
</tr>
</tbody>
</table>

The contents of each tube are then diluted tenfold with the glycerol-phenol diluent. These dilutions are used as antigens in a tube agglutination test with the dilution of ISAbS (or its national counterpart) in the manner shown in Table 7, page 80.

The agglutination reactions are read after 48 hours' incubation at 37° C. The tube of the stained antigen dilution that shows 50% agglutination with the 1:500 dilution of the standard serum corresponds to the correct dilution of the stained antigen.

1 Bendtsen (1949).
2 Renoux (1952).
The next step, therefore, is to dilute the concentrated stained suspension to the same extent as the tube whose tenfold dilution has given the correct agglutination titre; i.e., the concentration in the tube before the tenfold dilution had been made. In this way a stained antigen is produced that is always uniform and standardized and that permits comparable results to be obtained.

For example, suppose that tube No. 2 in the series (diluted tenfold) gives 50% agglutination with the 1:500 dilution of the standard serum. Then to obtain the standardized stained antigen, 0.8 ml of diluent must be added to each millilitre of the initial concentrated suspension. Consequently, if there are 500 ml of initial concentrated suspension, 400 ml of diluent are added, giving a total of 900 ml of standardized stained antigen.

The ring test antigen should always be stored in the refrigerator but should not be frozen.

The ring test with cow's milk

Collection of milk samples

Milk samples may be collected in 14×100-mm test tubes containing 0.5 ml of formalin preservative.1 The milk contained in cans or holding tanks is thoroughly mixed so that the cream is evenly dispersed. A quantity of milk is obtained from each can of milk, but milk from no more than 3 cans should be pooled in each collection tube. When collecting from holding tanks, milk from no more than 1 holding tank should be put in each collection tube. A small metal dipper is commonly used for collecting milk from cans or a bulk tank. In order to avoid the carry-over of milk and possible false or suspicious reactions, the dipper should be thoroughly rinsed in water after sampling the milk of each herd. It is desirable that the samples be held under refrigeration for 48–72 hours before being tested.

Procedure for the milk ring test

Mix the milk sample thoroughly to disperse the cream evenly and transfer 1 ml into a narrow (11×100-mm) test tube. Add 1 drop (0.03 ml) of ring test antigen and while closing the top of the tube with the index finger, mix by gently shaking and inverting the tube several times.

Rinse and dry the index finger between samples. Allow the mixture to stand for about 1 minute and then examine it to make sure that the antigen is thoroughly mixed with the milk. Incubate at 37°C for 1 hour.

Reading the test. The test is read, using a uniform light source. If the intensity of blue (haematoxylin antigen) or red (tetrazolium antigen) colour in the cream layer is deeper than in the skim portion, the test is

---

1 Prepared by mixing 7.5 ml of 37% formalin (commercial grade) with 1 litre of distilled water.
considered positive. If the intensity of colour in the cream layer is equal to or less than in the skim portion, the test is considered negative.

Adjustment of the Brucella ring test for herd size

When milk is collected in bulk tankers, it is desirable to adjust the BRT to compensate for the dilution factor. In California, USA, the BRT on milk from bulk tankers is adjusted according to herd size as follows:

<table>
<thead>
<tr>
<th>No. of animals</th>
<th>Volume of milk for BRT</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤200</td>
<td>1 ml</td>
</tr>
<tr>
<td>201-500</td>
<td>2 ml</td>
</tr>
<tr>
<td>501-900</td>
<td>3 ml</td>
</tr>
</tbody>
</table>

When there are more than 900 animals, it is recommended that the herd be broken down into smaller segments for the purposes of collecting milk for the BRT.

When 2 or 3 ml of milk are used the BRT is conducted in 14×100-mm tubes. Pooled negative cream (0.1 ml), collected as described on page 123, is added to each tube, followed by 1 drop (0.03 ml) of ring test antigen. After mixing, the test is incubated and read in the same way as for the milk ring test described above.

Serial dilution Brucella ring test

It is possible to detect infected individuals in a herd by using the serial dilution BRT, but the test is generally used to supplement the serological tests when questionable results are obtained.

A composite sample of unpasteurized milk from a herd of 25 or more cows that is negative to the BRT is used as the diluent.

A screening test is conducted on an animal by taking a milk sample (approximately equal quantities from each quarter), delivering 0.5 ml into an 11×100-mm test tube, adding 0.5 ml of composite negative milk, adding 1 drop (0.03 ml) of antigen, incubating at 37°C for 1 hour and reading. If a positive result is obtained, individual quarter samples are tested as follows:

Set up 4 rows of 11×100-mm or similar tubes, one row for the samples from each quarter and 10–12 tubes in each row. Add 1 ml of negative composite milk to each tube to act as diluent. Add 1 ml of the sample to be tested to the first tube of the row and mix with the negative milk already there; then transfer 1 ml of the mixture to the second tube and so on, 1 ml being discarded from the last tube in the row. When the samples from each quarter have been diluted add 1 drop (0.03 ml) of antigen to each tube, mix, incubate at 37°C for 1 hour, and read. The highest dilution in which a positive reaction occurs is the end titre for that quarter. A titre of 1:16 or more can often be correlated with the presence of Brucella organisms in the milk.
Ring tests carried out on undiluted milk samples from individual cows may give false-positive results shortly after parturition, near the end of lactation, and when mastitis is present.

**The ring test with cow's cream** (Pietz et al., 1967)

*Reagents required*

1. Diluent—prepare 150 ml of saturated sodium bicarbonate, add 7.5 ml of formaldehyde, and add water to bring the volume to 1 litre.

2. Neutralizer—prepare a 1 mol/litre solution of sodium bicarbonate in 0.85% NaCl and mix with an equal quantity of evaporated milk.

3. Pooled negative cream—obtain untreated milk from a brucellosis-free herd containing at least 25 lactating cows. Add 1 ml of formaldehyde to each litre of milk, mix, and allow the cream to rise by storing in a refrigerator overnight. Remove the cream.

4. Ring test antigen—haematoxylin-stained ring-test antigen is prepared and standardized as described on pages 118-119.

*Collection of cream samples*

Mix the can of cream thoroughly before sampling. Deliver approximately 8 ml of the cream sample into 14 × 100-mm tubes containing 1.2 ml of diluent. Insert the stopper and mix the cream and diluent by inverting several times. If possible, store under refrigeration until tested.

*Test procedure*

Allow the cream samples to warm to room temperature and centrifuge at approximately 1000 g for 15 minutes. Attach a 15-gauge canula, which is slightly longer than the 100-mm tubes, to a 2-ml rhemeter syringe and set the syringe to deliver 1.2 ml. Depress the plunger of the syringe and insert the canula along the inside edge of the tube through the cream layer. Withdraw from the skim portion of the sample by releasing the plunger. Deliver this portion into a 14 × 100-mm tube containing 0.6 ml of neutralizer. Mix and add 0.4 ml of pooled negative cream and 1 drop (0.03 ml) of ring-test antigen. Mix thoroughly and incubate at 37°C for 1 hour. The test is read in the same way as the milk ring test, which is described on page 121.

**The ring test with sheep's and goat's milk**

The test is carried out by adding one drop (0.03 ml) of antigen, either haematoxylin or tetrazolium stained to 1 ml of milk as is done with cow's milk. With the milk of sheep and goats positive reactions are manifested by agglutination of the antigen which usually falls to the bottom of the
tube leaving the milk column white. Occasionally, however, agglutinated antigen rises to the surface with the fat globules producing a ring as occurs with cow's milk; clumps of agglutinated antigen occasionally remain dispersed in the milk column. In negative reactions there is no change in the appearance of the milk column; it remains uniformly blue or red according to the antigen used. The tubes should be incubated at 37° C for 3 hours before the results are read.

Serological cross-reactions

Serological cross-reactions with Brucella have been reported in cases of infection or vaccination with some strains of Vibrio (Campylobacter), Pasteurella, or Salmonella.

Recently, strong cross-reactions in both the agglutination and complement fixation tests have been reported between smooth species of Brucella and Yersinia enterocolitica serotype 9 (Ahvonen et al., 1969). These have been attributed to similarities on the O-specific side chains of the lipopolysaccharide molecule of the two organisms (Hurvell & Lindberg, 1973). In contrast, protein antigens that lack lipopolysaccharide show no cross-reactivity in gel precipitation tests with hyperimmune sera (Diaz et al., 1970).

As Y. enterocolitica serotype 9 is frequently isolated in cases of human yersiniosis in Europe and this serotype has also been isolated from pigs, dogs, and cats (Winblad, 1973), the possibility of yersiniosis interfering with routine serological tests for brucellosis should be kept in mind. At present, there are no simple methods for the serological differentiation of the two infections. Cross-agglutinin absorption of sera is troublesome and not entirely definitive. Precipitating antibodies to the specific protein antigens are not normally present in infected sera in sufficient concentration to be detected by a gel diffusion test.
CHAPTER 3

ALLERGIC TESTS

Introduction

Hypersensitivity to Brucella antigens is acquired by man and animals during the course of infection, following vaccination, or following exposure to Brucella organisms or killed antigens in the laboratory. Cutaneous hypersensitivity, as demonstrated by the erythematous response to intradermal injection of antigen may be mediated by immunoglobulins, by immune cells, or both.

Reactions due to immunoglobulins are termed immediate-type hypersensitivity. If cytotoxic antibody is present, wheal and erythema develop within 20 minutes. If circulating precipitating antibody is present, oedema, erythema, and haemorrhage may develop 2–5 hours after intradermal injection of antigen and may persist for 24 hours or longer.

The delayed-type hypersensitivity reaction is mediated by sensitized thymus-derived (T) lymphocytes. Erythema may appear within 6 hours but the maximum erythema and induration occurs 24–48 hours after injection.

Although the delayed-type reaction is often assumed to be the one involved in allergic tests employed for diagnosis, it is probable that serum-mediated reactions are also evoked in most cases. The intensity of the immediate-type reactions varies with the allergen employed and the history of sensitization of the individual and may interfere with the interpretation of the delayed reaction. In the standardization of allergens in experimental animals, it is particularly important to be aware of the possibility that serum-mediated reactions may occur.

Use and limitations of allergic tests

The similarity of Brucella hypersensitivity to that of tuberculin sensitivity prompted the use of allergic tests for the diagnosis of brucellosis. Despite the development of a variety of skin test antigens over the last 50 years, there is no general agreement on the value of the test among medical or veterinary workers concerned with brucellosis.

It is agreed that a positive allergic test with a standardized Brucella allergen indicates that an invasion of the tissues by Brucella has occurred, but it does not prove the presence of active disease. Individuals can retain
sensitivity for a long time after clinical symptoms disappear. Sensitivity may be acquired following subclinical as well as clinical brucellosis.

A negative allergic test is no proof that the individual is not infected. Some individuals have shown negative tests at stages of infection when Brucella could be isolated. The history, clinical symptoms, and serological results must all be taken into consideration in the interpretation of an allergic test.

Vaccination produces sensitivity to allergic tests that may persist for a long time. In domestic animals, killed vaccines containing adjuvant produce sensitivity as persistent as that produced by living attenuated vaccines. The use of allergic tests may be limited, therefore, to non-vaccinated individuals.

One of the features of allergic tests is that serum antibodies may increase in titre following the test. Even allergens that are purified and produce no agglutinin response in normal individuals may stimulate an anamnestic response in individuals previously exposed to Brucella. This humoral response has been demonstrated not only with the agglutination test, but also with complement fixation and antiglobulin tests.

**Allergic tests in the diagnosis of human brucellosis**

The test is performed in man by injecting 0.1 ml of the allergen intradermally into the forearm. The reaction is read 24 and 48 hours later. Positive reactions are indicated by circumscribed areas of erythema, oedema, and induration that vary in diameter from 2 to 10 cm or more and may persist for as long as 7 days.

Extremely severe skin reactions, including sterile abscesses, occasionally occur in individuals with hyperery, even with carefully standardized antigens. In addition to a long-lasting local reaction, highly sensitized persons may show a rise in temperature, headache, indisposition, lymphadenitis, and lymphangitis. In some cases the entire clinical picture of brucellosis can be reproduced following a skin test. It is questionable whether any allergic tests should be used among veterinarians and laboratory workers who are constantly exposed to the organism.

In cases of obscure fever or undiagnosed chronic illness, serological tests for brucellosis should be carried out first. If these are positive, a skin test would be of no added value and might be harmful. If serological tests are negative or of low titre, an allergic test may be helpful. A negative allergic reaction and no rise in titre to serological tests following the allergic test can help to rule out brucellosis as a cause of the illness. A positive allergic reaction and a possible rise in titre must always be interpreted in terms of the patient’s history, since persons may show allergic cross-reactions because of other diseases. The availability of new protein allergens may overcome this problem.
Although allergic tests may be of debatable value in the diagnosis of human brucellosis, they are recommended for use in individuals with no known history of brucellosis who are to be given a *Brucella* vaccine. In some countries, occupational groups that anticipate heavy exposure to *B. melitensis* infection are vaccinated with living *B. abortus* strain 19-BA vaccine if their intradermal test is negative.

**Allergic tests in domestic animals**

Allergic tests are employed in some countries for the detection of brucellosis in sheep, goats, pigs, and cattle. In nonvaccinated populations, allergic tests are most useful as herd screening tests. Animals on farms with no history of exposure to infection and with negative serological tests are usually negative to allergic tests. Conversely, farms with animals positive to allergic tests usually have some animals with positive serological tests or have a history of overt infection. Allergic tests cannot be relied upon for diagnosis of infection in individual animals. Vaccinated animals, or those that have cleared themselves of natural infection, may remain allergic reactors for a long time, and animals that are shedding organisms in milk or genital secretions may be negative to allergic tests.

In countries in which an allergic test has been employed for diagnosis in sheep, it is recognized that its use alone, without accompanying serological tests, will not result in eradication of the disease from flocks. Infected sheep frequently do not react to an allergic test until after lambing or abortion, and thus the disease spreads before it is detected. Sheep suffering from malnutrition respond poorly to allergic tests.

Allergic tests are very useful in epidemiological surveys, particularly in areas with a low incidence of brucellosis where the number of serologically positive animals is low, or where serological testing is impractical. It offers the advantage that application is possible on a large scale.

Allergic tests are employed in some countries, together with serological tests, as a control measure for imported animals. In animals that develop serological reactions between the country of origin and the port of entry, an allergic test may help to clarify the diagnosis.

Several procedures for the inoculation of *Brucella* allergens have been employed, e.g., intradermally (0.1 or 0.2 ml) into the caudal fold of cattle, sheep, and goats, intradermally into the dorsal surface of the ear of pigs and goats, intradermally into the neck or flank region of cattle, subcutaneously (0.5 ml) or intradermally (0.1 ml) into the upper or lower eyelid. The palpebral route has been found by a number of workers to be the preferred method for sheep and goats. It is convenient to inject and easy to read, as the swelling in the injected eyelid is readily apparent in comparison with the other eye.
Nature of the allergens

The ability of proteins to elicit specific delayed hypersensitivity reactions is generally recognized. The role of polysaccharide and lipopolysaccharide antigens in delayed hypersensitivity reactions is debatable, although these antigens are clearly involved in the immediate antibody-mediated cutaneous reactions.

*Brucella* allergens are generally a mixture of protein and polysaccharide or lipopolysaccharide antigens, the actual composition depending upon the method of preparation and the culture employed. Since animals infected with *Brucella* usually have serum antibodies to polysaccharide determinants of the smooth cell wall lipopolysaccharide, it is probable that both antibody-mediated and delayed-type reactions are elicited by most allergens.

The lipopolysaccharide component of *Brucella*, if present in sufficient quantities, will produce induration, erythema, and necrosis at the skin test site of normal animals and will induce antibody formation. The presence of lipopolysaccharide antigens, even in small quantities, is responsible for the anamnestic rise in agglutinins following the allergic test.

As it is difficult to prepare protein antigens that are completely free of traces of lipopolysaccharide antigens, some workers have employed non-smooth *Brucella* cultures for the preparation of allergens in order to eliminate the lipopolysaccharide component. Protein allergens prepared from rough and smooth cultures of *Brucella* showed the same specificity in guineapig skin tests and did not cause reactions in normal guineapigs (Jones et al. 1973b; Jones, 1974).

An examination of the literature shows that few *Brucella* allergens have been characterized for their chemical composition or for the type of hypersensitivity that they elicit. Many allergens are called "Brucellin" although they vary in composition from whole cells to extracts. In general, four types of preparation have been employed.

1. Killed whole cells have been used as allergens but they are no longer recommended because they stimulate an antibody response following skin test.

2. Crude culture filtrates or supernatant fluids have been employed. "Melitin", a well-known example of this type, has been described by Alton & Jones (1967). It produces toxicity reactions in normal animals and the method of standardization previously described is no longer recommended.

3. A number of preparations have been produced by hydrolysis of cells by heat treatment with acid. The "brucelhydrolysate" described by Ivanov and cited by Alton & Jones (1967) is no longer employed in the USSR. It has been superseded by "brucellizate".

4. Water or saline extracts of *Brucella* cells are widely used in methods of preparation. Cells are disintegrated before extraction in the methods for
"brucellergen" (Huddleston, 1943), for MPB antigen (Castañeda, 1953, cited by Alton & Jones, 1967) and for "brucellizate" (Ivanov unpublished, 1962). Whole cells are extracted in cold saline in the method of Bhongbibhhat et al. (1970).

Production and use of the allergens

Brucellizate

The method of preparation of "brucellizate" as given by Ivanov (unpublished report, 1962) is as follows: cells of a smooth strain of \textit{B. suis} are dried, ground with steel balls for 18–24 hours, then extracted with saline at pH 8.0 in the cold for 1–3 days, sedimented and the liquid is sterilized by Seitz filtration. The final product is standardized by nitrogen content to 8–10 mg/100 ml. The activity and specificity is checked simultaneously with a control preparation in infected and normal sheep or in guineapigs.

Brucella protein allergen

A protein-containing allergen, with no detectable lipopolysaccharide antigen, has been developed and characterized (Jones et al., 1973b) based on the extraction procedure described by Bhongbibhhat et al., (1970). A rough strain of \textit{B. melitensis}, strain B115, is employed to avoid the presence of lipopolysaccharide in the allergen. The bacteria may be grown on agar, or in aerated broth cultures. The bacteria are harvested, washed 3 times in saline, and suspended in normal saline to give a concentration of about $1\times10^{10}$ cells per ml. The bacterial suspension is added to 2 volumes of acetone at $-20^\circ$ C, and is allowed to stand at $4^\circ$ C for 18 hours. The bacteria are then sedimented by centrifugation at $4^\circ$ C, washed 3 times with cold acetone and dried under vacuum in a desiccator. The dried bacteria are suspended in 2.5% sodium chloride solution at $4^\circ$ C to give a 5% w/v suspension. (All subsequent handling is carried out at $4^\circ$ C until the final product is dry.) The suspension is agitated for 3 days, then centrifuged to sediment the bacteria and the supernatant is treated with 3 volumes of cold ethanol during constant mixing on a magnetic stirrer. The mixture is held for 24 hours, then centrifuged to separate the precipitate that forms. The supernatant is discarded and the sedimented precipitate is dissolved in water and then dialysed against distilled water in the cold. The solution is centrifuged at 100 000 g for 6 hours to remove high molecular weight material and the supernatant fluid is removed and freeze-dried.

The allergen contained 50% protein when tested by the Folin–Ciocalteu method (Williams & Chase, 1968). Spectrophotometric analysis in a 1-cm cuvette at a concentration of 1 mg dry allergen per ml of sodium phosphate buffer (pH 7) showed greater absorption at 260 nm than at 280 nm. This indicated that a significant amount of nucleic acid material was present as
well as protein but this did not affect the allergic reaction. The skin test activity of the allergen resisted heating at 100° C for 30 minutes and treatment with ribonuclease, but was destroyed by pronase. Gel filtration in Sephadex G-75 showed that the skin test active fractions were composed of a mixture of proteins with molecular weights in the range of 12,000–50,000.

Lipopolysaccharide antigen could not be detected in the allergen by immunological methods. Hyperimmunization of rabbits with the allergen in Freund’s incomplete adjuvant did not result in the production of agglutinins, precipitins, or complement fixing antibodies to smooth or rough cell wall antigens, but stimulated production of precipitins and complement fixing antibodies to the protein allergen. Extensive studies in guineapigs, rabbits, sheep, and cattle previously sensitized with Brucella organisms or vaccines, have shown that the use of the allergen does not result in an anamnestic rise of antibodies to smooth cell wall antigens. Repeated injection of the allergen into normal animals does not result in either sensitivity to the allergen or antibody production to smooth cell wall antigens. The use of the allergen, therefore, does not interfere with subsequent testing.

Titration of allergens

A standard method for the titration of Brucella allergens has not been agreed upon. The use of allergens in excessive concentration is extremely hazardous to human health. Pharmaceutical companies and government laboratories that have produced allergens by the same method for many years have developed their own system for producing a safe product. Until methods for standardization have been established, laboratories that have not had extensive experience in the preparation of allergens should not employ their preparations on human subjects.

Standardization of allergens on sensitive persons is not advised as it can lead to serious illness.

The guineapig is the best laboratory animal for the demonstration of delayed hypersensitivity reactions. Guineapigs can be used to determine the potency of an unknown allergen, to compare potencies of several allergens, and to standardize successive batches of allergen. The optimum dose for veterinary diagnosis, however, can be determined only by direct testing in the species concerned.

Use of guineapigs for standardization and comparison of allergens

Albino guineapigs should be maintained on a ration that includes a daily source of vitamin C and should weigh at least 350 g prior to sensitization.

The method of sensitization and the total dose of allergen injected intradermally per animal are the most important factors influencing the results (Jones et al., 1973a).
Method of sensitization

Guineapigs infected with the attenuated strain, *B. melitensis* strain Rev. 1, are sensitive 1 week after infection and give good skin test reactions from 2 to 10 weeks after infection. Guineapigs infected with a virulent *Brucella* strain (e.g., *B. abortus* strain 544) show very poor delayed hypersensitivity reactions for the first 4 weeks of infection, but are very sensitive to small amounts of allergen from 10 to 28 weeks after infection. The use of an attenuated strain rather than a virulent strain offers advantages in safety as well as in the shorter time interval required between sensitization and skin testing. For these reasons the recommended method for sensitization of guineapigs is the subcutaneous inoculation of $10^8$ organisms of the attenuated strain *B. melitensis* strain Rev. 1.

Dose of allergen for intradermal test

The response to any given dose of allergen is depressed if guineapigs are given other injections of allergen simultaneously. Therefore, the injection of serial dilutions of allergen in the same animal may not give a true indication of the potency of the allergen. The optimum dose may be more accurately defined if a series of 10-fold dilutions is given to groups of guineapigs in which each animal is given a single dose of allergen. This method has shown that the largest dose does not necessarily give the largest response.

Determining the optimum dose of an unknown allergen for the intradermal test

The concentration of the allergen should be expressed in one or more of the following ways:

(a) dry weight (microgram quantities or less are sufficient to evoke skin reactions in optimally sensitized guineapigs).

(b) protein estimation by ultraviolet spectrophotometric analysis at 280 nm (Williams & Chase, 1968).

(c) protein concentration by the Folin–Ciocalteu reaction (Williams & Chase, 1968).

Prepare 4 or 5 tenfold dilutions in sterile pyrogen-free saline for the preliminary titration. Label a separate syringe for each dilution. Use 1 ml disposable tuberculin syringes fitted with 26-gauge short-bevel needles.

For the preliminary titration, groups of 3 sensitized and 2 or 3 normal guineapigs are sufficient for each dilution. Guineapigs should be sensitized by the subcutaneous inoculation of $10^8$ organisms of the attenuated strain *B. melitensis* Rev. 1 two to ten weeks prior to the skin test. The hair on one side of each guineapig is removed with a suitable small-animal clipper.¹

¹ The most widely employed small-animal clipper is obtained from John Oster Manufacturing Co., Milwaukee, Wisc., USA. Model A5 clipper with Size 40 blade.
Each animal is injected intradermally with 0.1 ml of one of the dilutions to be tested. At 24 hours the opposing diameters of erythema at each injection site are measured with vernier calipers and the mean diameter of erythema for each dilution is calculated. Higher doses may produce less response than lower doses. For example, 1 µg may evoke a 17-mm reaction (mean diameter), 0.1 µg and 10 µg may evoke 11-mm reactions, and 100 µg may produce no erythema. A dose of 1 µg would be selected as the quantity about which a second titration should be carried out within a few days in guineapigs from the same sensitization group, but not in the same individuals. Three doses, e.g., 0.1, 1, and 10 µg would be given to groups of 5 or more sensitized guineapigs, a single dose per animal. The second titration should establish the optimal reacting dose for the allergen, that is the dose giving the largest mean reactions at 24 hours. Normal guineapigs given 100 x the optimal reacting dose should show no reaction.

Comparison of allergens in guineapigs

When the protein concentration and the optimal reacting dose for guineapigs have been established for a given preparation, it is possible to compare successive batches. The protein concentration of the new batch should be adjusted so that it is identical with the standard preparation. If guineapig titrations show that the two preparations have approximately the same reactivity when given as single doses per animal, a direct comparison of the two preparations can be made by simultaneous injection on the same animals of several dilutions of each preparation. The total dose of allergen injected per animal should approximate the optimal dose of allergen for animals of that sensitization group, e.g., if 1 µg is the optimum dose of each of two allergens to be compared, then 3 dilutions containing 0.5, 0.1, and 0.02 µg of each allergen could be tested simultaneously.

Titration of allergen in domestic animals

After the protein concentration of the allergen has been determined and the potency established in guineapig skin tests, the optimum dose of allergen for testing cattle, sheep, goats, or pigs must be determined in the species concerned. Tenfold dilutions of the preparation are injected by the desired route into normal and sensitized animals, using a single dose per animal.

When the relationship between the optimum dose for guineapigs and that for large animals is established, it should be possible to rely on potency testing in guineapigs for standardization of successive batches of allergen.
CHAPTER 4

THE PRODUCTION OF *BRUCELLA* VACCINES

Introduction

Two vaccines are in general use for immunizing cattle against brucellosis—the living attenuated *B. abortus* strain 19 vaccine, and the killed *B. abortus* strain 45/20 adjuvant vaccine.

Strain 19 vaccine may produce persistent serological reactions in cattle, especially when they are vaccinated as adults; moreover, it may cause pregnant cattle to abort, and for these reasons it should be administered only to calves between 3 and 6 months old, or at the latest, at 8 months of age. The resistance produced by vaccination at this age lasts for many years and the resulting serological reactions usually fall below diagnostic levels before first calving. The method for producing strain 19 vaccine as recommended by the United States Department of Agriculture is given in detail below.

The killed *B. abortus* strain 45/20 adjuvant vaccine can be given to cattle of any age, whether pregnant or not. It does not usually produce levels of agglutinins likely to interfere with diagnostic agglutination tests, but does cause the production of antibodies that interfere with the complement fixation and Coombs tests. It should be noted, however, that the administration of strain 45/20 adjuvant vaccine to animals that have previously been vaccinated with strain 19 may result in a rise in agglutination titre above 30 IU's and its use in these circumstances can interfere with diagnostic agglutination tests. It also has the disadvantage that repeated vaccination is necessary and that undesirable reactions occasionally occur at the site of inoculation. The production of this type of vaccine is almost entirely in commercial hands but many of the details of production have been published (Roerink, 1966).

The living attenuated *B. melitensis* strain Rev. 1 is widely used to immunize sheep and goats against brucellosis caused by *B. melitensis*. The Rev. 1 vaccine, when used in the dosage originally recommended, had the same disadvantages as the *B. abortus* strain 19 vaccine. Recent research (Alton, 1969, 1970; Alton et al., 1972; Jones et al., 1973c) has shown, however, that in goats a much reduced dose of Rev. 1 vaccine is still capable of producing immunity but does not stimulate the production of undesirable antibody levels, and is not capable of causing abortion; thus a reduced dose may be used to vaccinate adult goats. A detailed description of the production of Rev. 1 vaccine is given later in this chapter.

—133—
Brucella abortus strain 19 vaccine

*B. abortus* strain 19 was originally isolated in 1923 and was reported by Buck (1930) to be of reduced virulence and effective as a vaccine for cattle. This strain has a stable low pathogenicity, a relatively high immunogenicity and does not spread from animal to animal. These characteristics have been maintained by the careful selection of seed cultures, the preparation of an original seed with the desired properties for vaccine production, and preservation of this in the freeze-dried form. Proper control in the production and use of this vaccine is necessary to ensure that the product has the stability, safety, and effectiveness it has generally shown over the years. Requirements for *B. abortus* strain 19 vaccine (live—for veterinary use) have been described by the WHO Expert Committee on Biological Standardization* and satisfactory original seed in a form for preparing vaccine or for preparing seed lots is available.* It is recommended that, in those countries in which strain 19 vaccine is produced by more than one laboratory, a central laboratory should obtain and distribute original seed cultures or obtain original seed cultures and prepare freeze-dried seed cultures for distribution to the other laboratories. In addition, the vaccine produced by each laboratory should be tested by the central laboratory before being released for use.

Strain 19 vaccine may be produced either on a solid medium or by the more recently developed batch method, which utilizes a liquid medium. Potato agar (page 15) has been found to be a most satisfactory solid medium for maintaining the stability of strain 19 and is recommended for its growth. However, other solid media are satisfactory, provided neither serum nor animal tissue is incorporated in the medium. Since growth on moist media may result in dissociation, it is desirable to incubate both slopes and Roux flasks of medium until there is no condensation water present (usually about 2-4 days). Phosphate-buffered saline, pH 6.3 is used as the diluent throughout the preparation of strain 19 vaccine. A concentrated stock solution may be prepared as follows: dissolve 50 g of sodium dihydrogen phosphate dihydrate (NaH₂PO₄·2H₂O), 22 g of anhydrous disodium hydrogen phosphate (Na₂HPO₄), and 830 g of sodium chloride in 10 litres of distilled water. This concentrate is diluted 1:10 in distilled water and sterilized before use. If the vaccine is to be freeze-dried it should be harvested in a stabilizer for drying (page 20).

It is advisable to protect the gauze-wrapped cotton plugs used in flasks and bottles by covering the entire neck with paper prior to sterilization.

It is convenient to use an aspirator bottle fitted with a protective bell filling attachment for dispensing buffered saline and stabilizer, for seeding Roux flasks, and for dispensing the final bulk into vials (Fig. 13, page 67).

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*Available through the World Health Organization or directly from the United States Department of Agriculture, Veterinary Services Laboratories, P.O. Box 70, Ames, Iowa 50010, USA.
In order to minimize the danger of contaminating the vaccine during preparation, procedures such as transferring cultures, seeding and harvesting Roux flasks, and vaccine bottling, should be carried out in booths that can be thoroughly scrubbed with a disinfectant beforehand, that have a minimum of cross draughts, and (ideally) that are fitted with air-filtering devices and ultraviolet lamps. Personnel should wear protective clothing.

**Propagation and harvesting of strain 19 on potato agar medium**

**Preparation of medium**

Prepare potato agar as described on pages 15 and 16 and dispense it in Roux flasks (for production) and culture tubes (slopes for seed and purity testing). The tubes for seed slopes should be at least 20 x 150 mm in size and they should be set at an angle while the medium is hardening so that the sloped surface of the medium is at least 50 mm long. After the medium has solidified, incubate at 37°C until the surface is dry (usually 2–4 days).

**Preparation of seed material**

Restore a freeze-dried original seed culture¹ or a freeze-dried seed culture prepared from original seed by the central laboratory and inoculate it on to a number of potato agar slopes, contained in culture tubes that are at least 20 x 150 mm in size, making sure the entire surface of each slope is inoculated. Incubate at 37°C for 48 hours. Examine each slope culture visually and discard those that are contaminated. The seed slopes are stored at 4°C and may be used as seed material for up to 3 months provided excessive drying does not occur.

**Preparation of a single harvest**

Add about 10 ml of phosphate-buffered saline (or sufficient to submerge the slope), pH 6.3 (prepared as described on page 134) to each culture tube containing a seed slope and allow to stand for 15–20 minutes; then wash the brucellae off the medium by rolling each tube through the hands. Pour this seed material aseptically into phosphate-buffered saline contained in an aspirator bottle equipped with a protective bell filling attachment (Fig. 13, page 67). Use the growth from 1 slope for each 200 ml of phosphate-buffered saline. Aseptically inoculate each Roux flask containing potato agar with 5–10 ml of seed material contained in the aspirator bottle. Rotate each Roux flask so that the inoculum is spread over the entire surface of the medium and incubate the Roux flasks in an inverted position at 37°C for 48 hours. Examine each Roux flask visually and discard any that are contaminated. Aseptically pour off and discard the residue of seed material from each satisfactory Roux flask and introduce about 25 ml of phosphate-

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¹ Available through the World Health Organization or directly from the United States Department of Agriculture, Veterinary Services Laboratories, P.O.Box 70, Ames, Iowa 50010, USA.
buffered saline (for liquid vaccine) or stabilizer (for freeze-dried vaccine) from an aspirator bottle equipped with a protective bell. With the medium in a downward position, allow each Roux flask to stand for not more than 15–20 minutes and then gently rock each flask until the growth is in suspension.

Aseptically pour off the suspension from a number of Roux flasks into a sufficiently large Erlenmeyer flask, conduct tests for identity and for absence of contaminating organisms on each pooled suspension, and hold each pooled suspension at 4°C. Discard all suspensions found to be unsatisfactory. All satisfactory suspensions that were inoculated on the same day are pooled into a single harvest, which is stored at 4°C and used for preparing vaccine. Tests for identity and for absence of contaminating organisms are conducted on each single harvest. Further processing is described later under “Preparation of final bulk”.

Propagation and harvesting of strain 19 in liquid medium (batch method)

The fermentor equipment and medium are prepared in the same way as described for propagating B. abortus strain 1119-3 on pages 68–69.

Preparation of seed material

Seed material is prepared on slopes, as described on page 135, which are stored at 4°C and may be used as seed material for up to 3 months provided excessive drying does not occur.

Preparation of a single harvest

Inoculate the fermentor and incubate as described on page 69. After 48 hours incubation aseptically remove a sample and conduct tests for identity, for the absence of contaminating organisms, and for dissociation. Concentrate the Brucella cells from the medium by centrifugation, as described on page 70, or by adding 1.25 g of sodium carboxymethyl-cellulose per litre of suspension and storing at 4°C until the cells have settled out. Decant the medium. Suspend the concentrated Brucella cells in phosphate-buffered saline (liquid vaccine) or stabilizer (freeze-dried vaccine).

The suspension of Brucella cells from a fermentor is a single harvest. Store the suspension at 4°C until it is used for preparing vaccine. Tests for identity and for absence of contaminating organisms are conducted on each single harvest.¹

Preparation of final bulk

The final bulk is the finished material present in 1 container (an aspirator bottle with a protective bell is recommended) from which the final containers of a batch of vaccine are filled. The final bulk can be a single harvest or a pool of a number of single harvests that are derived from the same seed-lot.

¹ Methods are described on page 138.
The number of Brucella organisms per ml of the final bulk suspension is determined, using one or more of the enumeration methods described (pages 59–60). The volume of the final bulk is adjusted by adding sufficient phosphate-buffered saline (liquid vaccine) or stabilizer (freeze-dried vaccine) so that a dose (5 ml) contains between $40 \times 10^6$ and $120 \times 10^6$ viable organisms, or as specified by the national or regional control authority.

When freeze-dried vaccine is being prepared the bacterial concentration is standardized to allow for mortality during the freeze-drying operations. The degree of mortality can be determined when a standard drying technique has evolved for a particular apparatus, but allowance for mortality of about 50% would be a good starting point.

After adjusting the cell concentration of the final bulk, tests for identity and absence of contaminating organisms are conducted.

**Filling the vaccine containers**

The vials and rubber stoppers to be used are sterilized separately by autoclaving for 30 minutes at 120°C. It is essential that sterilization be carried out in such a manner that the vials are free from moisture.

For liquid vaccine, the final bulk suspension contained in 1 container such as an aspirator jar with a protective bell is dispensed aseptically into single-dose (5 ml) or multiple-dose vials that are stoppered with rubber stoppers and sealed with aluminium caps. If the material is to be freeze-dried, split rubber stoppers are partially inserted, the material is freeze-dried (see below), the split rubber stoppers are completely inserted, and the vials are sealed with aluminium caps.

**Freeze-drying (lyophilization) of strain 19 vaccine**

It is desirable to prepare strain 19 vaccine in the freeze-dried form as in this form it maintains a satisfactory viable count much longer than it does as a liquid. The final vials containing strain 19 suspended in stabilizer, and in which the split rubber stoppers have been partially inserted, are put into sterile trays and freeze-dried. The drying cycle should be arranged so that, at its conclusion, the temperature of the product does not exceed 35°C, and the residual moisture content is 1–2%. The freeze-dryer should be equipped with an internal stoppering system and the vials should be sealed under vacuum at the end of the drying cycle. Given these conditions, good stability of the vaccine can be confidently anticipated if it is stored at 4°C.

**Control tests on final vaccine**

Control tests are conducted on vials of vaccine that were filled towards the beginning and end of the filling process. With freeze-dried vaccine, the
control tests should be carried out on the vaccine reconstituted to the form in which it is to be used.

Tests for identity

Tests for identity are conducted on samples from each filling lot.

(a) Prepare a smear on a glass slide and stain by Gram’s method. The morphology of *Brucella* is described on page 34.

(b) Inoculate potato agar slopes, incubate at 37° C for 4 days, and observe the characteristic appearance.

Tests for absence of contaminating organisms

Test each filling lot for bacterial and fungal contamination by inoculating 2 tubes of dextrose Andrade’s broth (see page 19) containing inverted ampoules. Incubate at 37° C and examine for the presence of acid (red colour), gas (air in inverted ampoule), or both, at 2, 4, and 7 days.

Tests for dissociation

Culture 1 or more samples of each filling lot on potato agar, or other suitable medium, and examine the culture by obliquely reflected light, as described on page 37. The filling lot passes the test if not less than 95% of the colonies are of the smooth variety.

Test for number of viable organisms

Determine the number of viable organisms per millilitre of each filling lot by the USDA method (page 59) or by the Miles and Misra method (page 60).

Test for reactivity in guineapigs

Tests for antigenicity and immunogenicity

Test for stability

Test selected filling lots for stability by a method approved by the national or regional control authority, such as testing for the number of viable organisms, as described above, before and after the samples have been held at appropriate temperatures for appropriate periods. As a guide to stability some manufacturers determine the residual moisture content of the final lyophilized vaccine. The method of determining the moisture content should be one approved by the national or regional control authority.

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1 These tests are optional. A detailed description of methods in use for such tests is given in the *British veterinary codes: Supplement* 1970. There is some doubt about the effectiveness of these methods (Thornton & Muskett, 1972) and changes in the current recommendations of the *British veterinary codes* may be expected.
Administration of strain 19 vaccine in the field

Dried vaccine is reconstituted in the appropriate quantity, normally 5 ml of distilled water per dose, and must be injected within 2 hours of reconstitution. The vaccine is administered subcutaneously just behind the shoulder.

Differentiation of strain 19 from virulent field strains of Brucella abortus

Strain 19 has all the characteristics of B. abortus biotype 1, as given in Table 2 (page 40), but differs from other biotype 1 strains in the characteristics given below. Occasionally cultures that resemble strain 19 are recovered from vaccinated animals. Such cultures should be subjected to the following tests in parallel with seed cultures used for vaccine production.

Carbon dioxide requirement

Strain 19 does not require carbon dioxide for growth.

Growth in the presence of thionin blue

The growth of strain 19, B. abortus biotype 2, and that of an occasional strain of other biotypes, is inhibited in the presence of thionin blue at a final concentration of 1:500 000.

Growth in the presence of penicillin

The growth of strain 19, B. abortus biotype 2, and that of an occasional strain of other biotypes, is inhibited in the presence of penicillin at a concentration of 5 IU per ml of medium.

Growth in the presence of erythritol

The growth of strain 19 is inhibited in the presence of erythritol at a concentration of 1 mg per ml of medium.

Oxidative metabolic tests

The oxidative metabolic pattern of strain 19 on substrates recommended for species identity is typical of B. abortus; however, there are consistent quantitative differences between strain 19 and other strains of B. abortus in the oxidative rates on certain substrates (Brown et al. 1972), as follows:

(a) The oxidative rate (QO$_2$(N)) value of strain 19 on L-glutamate is consistently higher than that of other strains of B. abortus and is usually over 500.

(b) The oxidative rate of strain 19 on D-erythritol is consistently lower than that of other strains of B. abortus.

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1 Available from K. & K. Laboratories Inc., Rare Chemicals, Plainview, N.Y. 11803, USA.
(c) The oxidative rate of strain 19 on l-alanine is approximately double the rate on D-alanine.

(d) The oxidative rate of strain 19 on D-galactose is approximately equal to the rate on D-ribose.

The following oxidative rates were obtained on 30 serial lots (serial lots 55–84) of strain 19 seed cultures produced during 1956–68 at the Veterinary Services Laboratories, Ames, Iowa, USA.

<table>
<thead>
<tr>
<th>QOx(N) value</th>
<th>Mean</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-alanine</td>
<td>92</td>
<td>69–121</td>
</tr>
<tr>
<td>L-alanine</td>
<td>179</td>
<td>99–239</td>
</tr>
<tr>
<td>L-asparagine</td>
<td>206</td>
<td>154–292</td>
</tr>
<tr>
<td>L-glutamate</td>
<td>604</td>
<td>507–690</td>
</tr>
<tr>
<td>D, L-ornithine</td>
<td>89</td>
<td>30–124</td>
</tr>
<tr>
<td>D, L-citrulline</td>
<td>40</td>
<td>21–59</td>
</tr>
<tr>
<td>L-lysine</td>
<td>30</td>
<td>6–48</td>
</tr>
<tr>
<td>L-arginine</td>
<td>84</td>
<td>37–119</td>
</tr>
<tr>
<td>L-arabinose</td>
<td>132</td>
<td>55–198</td>
</tr>
<tr>
<td>D-galactose</td>
<td>244</td>
<td>176–305</td>
</tr>
<tr>
<td>D-ribose</td>
<td>250</td>
<td>210–316</td>
</tr>
<tr>
<td>D-erythritol</td>
<td>23</td>
<td>10–35</td>
</tr>
</tbody>
</table>

**Test for reactivity in guineapigs**

Strain 19 can be distinguished from virulent field strains of *B. abortus* by a test for reactivity in guineapigs that is based on the limited multiplication of strain 19 organisms in the spleen and on the agglutinin response in these animals.\(^1\)

**Brucella melitensis strain Rev. 1 vaccine**

Rev. 1 is a smooth attenuated strain of *B. melitensis* (Elberg & Faunce, 1957). It is a non-streptomycin-dependent clone isolated from a population of streptomycin-dependent cells that were in turn derived from the parental virulent strain 6056 of *B. melitensis*. The effectiveness of the Rev. 1 vaccine in protecting sheep and goats against *B. melitensis* infection is now well established.

Rev. 1 is liable to dissociation changes, as are other strains of *B. melitensis*, and special measures to control such changes are required in vaccine production. The WHO Expert Committee on Biological Standardization\(^2\) has recognized the need for control of *B. melitensis* Rev. 1 vaccine.

A satisfactory original seed of *B. melitensis* strain Rev. 1 in a form for preparing vaccine or for preparing seed-lots may be requested through the

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\(^1\) A detailed description of the method that is in use for this test may be obtained on application to the World Health Organization, 1211 Geneva 27, Switzerland.

World Health Organization or obtained directly from Professor S. S. Elberg, School of Public Health, University of California, Berkeley, Calif. USA.

Rev. 1 may be produced on a solid medium, as described below, or it may be grown in a liquid medium by a method similar to that described for the cultivation of B. abortus strain 19 in liquid medium on pages 136 and 137. For details of the cultivation of Rev. 1 in liquid medium see Garcia & Arce (1973).

Media for growth of strain Rev. 1

Trypticase-soy agar and Brucella agar (Albimi)¹ are amongst the media recommended for the growth and maintenance of strain Rev. 1. This strain does not grow well on potato agar. All media should be adjusted so that the final concentration of agar is at least 2%. Roux flasks of agar medium should be incubated for at least a week before being seeded. This has the effect of lessening the chances of dissociation and at the same time improving the yield of vaccine.

Preparation of seed cultures for local vaccine production

It is preferable to start each batch of vaccine from an original seed. If the number of batches of vaccine being produced makes this impracticable then dried seed lots may be produced locally. The second method given below for producing seed material may be used for producing dried seed lots. Two methods for the production of seed cultures for inoculating Roux flasks are given below because the appearance of rough variants in vaccine harvests has been a problem in some laboratories but not in others apparently using the same techniques.

Method 1. For use where the occurrence of dissociation has not proved to be a problem. The contents of an ampoule of original seed or locally produced seed lot are reconstituted and used to seed slopes of medium. After 48 hours’ incubation the growth is harvested in phosphate-buffered saline, pH 6.4; the contents from 4 large slopes may be diluted in about 100 ml of buffer.

Method 2. Where dissociation has proved to be a problem.

1. The contents of an ampoule of original seed or locally produced seed lot are reconstituted in distilled water and spread on 6 agar plates in such a way as to produce areas of confluent, semi-isolated, and isolated colonies; the plates are incubated for 5 days at 37°C.

2. Three of the plates inoculated with Rev. 1 are stained with a 1:2,000 solution of crystal violet as described on page 39, then examined with a hand lens or stereoscopic microscope. If dissociated colonies (in practice

¹ Available from: Chas. Pfizer & Co., New York, NY 10036, USA.
this could be taken to mean more than 0.1% are detectable in these cultures it must be considered that the seed material used to inoculate the plates was unsatisfactory and a new original seed should be obtained.

3. If no dissociated colonies are present on the 3 stained plates, a group of 5–10 colonies is transferred on to each of at least 6 agar slopes from the 3 unstained plates. These slope cultures are incubated for 2–3 days.

4. In order to test for dissociation in the slope cultures, take a loopful of growth from each, emulsify in approximately 0.5 ml of sterile saline and spread on agar plates so as to obtain isolated colonies. Incubate the plates for 5 days during which time the slope cultures are kept in the refrigerator. Test the growth on each plate for dissociation by emulsifying a few colonies in separate drops of dilute acriflavine solution (see page 38) and then staining the whole culture with crystal violet. Those slope cultures that produce completely smooth growth on the plates are used to make seed lots or to provide seed for the inoculation of Roux flasks for vaccine production. A pool from at least 4 slopes is required, the growth being harvested as in Method 1. For seed lots, the slope cultures are harvested in the stabilizer and then pooled and freeze-dried.

Preparation of vaccine

1. Each Roux flask for vaccine production is seeded with 2–5 ml of the suspension produced by either Method 1 or Method 2 described above. The flasks are then incubated for 3 days, after which the growth is harvested in the stabilizer (25 ml per Roux flask) that is to be used for freeze-drying or, if the vaccine is not going to be freeze-dried, it may be harvested in buffered saline at pH 6.4 (see page 20). The suspension from the Roux flasks is filtered through several layers of gauze, either by pouring or with suction, and the contents from a convenient number of flasks are pooled in aliquots.

2. Each aliquot of suspension is examined for contamination by inoculating both an agar slope and a fermentation tube containing dextrose Andrade’s broth. A count of viable cells is made, using one of the methods described on pages 59-60. This serves also as a check on dissociation (see section 3 below) and as a check on the identity of the suspension.

3. The vaccine suspension is stored at 0–5°C while the plates are incubating for the 5 days necessary for the growth of colonies, after which the cultures made for purity tests are examined. The colonies growing in the plates seeded for viability determination are counted before and after staining with crystal violet so that the viable count and the dissociation state may be determined. Up to 5% of nonsmooth colonies is permissible. Satisfactory aliquots are pooled to form a single harvest.

4. The rest of the procedure for producing Rev. I vaccine is identical to that described for strain 19 B. abortus vaccine under the sections entitled
“Preparation of final bulk”, “Freeze-drying of strain 19 vaccine”, and “control tests on final vaccine” (pages 136–138), except that an adjustment has to be made for the number of viable organisms required per dose of Rev. 1 vaccine. The number of Brucella organisms in the single harvest is already known and this needs to be diluted so that each dose for sheep and goats contains $1-2 \times 10^9$ organisms. For use in adult goats, Rev. 1 may, however, be dispensed in doses containing $10^5$ cells as discussed below under field use. The amount of diluent to be added when reconstituting dried Rev. 1 vaccine should be clearly stated on the bottle, together with the dose and the expiry date—normally 1 month for liquid vaccine and 1 year for dried vaccine. Distilled water, or preferably phosphate-buffered saline should be used for the reconstitution of dried vaccine.

**Characteristics of strain Rev. 1 that distinguish it from other strains of B. melitensis**

1. The maximum diameter to which colonies grow in 4 days at 37 °C is 1–2 mm. They are always smaller than other strains of *B. melitensis* grown under the same conditions.

2. The growth of strain Rev. 1 in air is inhibited by 1:50 000 thionin and 1:50 000 basic fuchsin. The presence of dyes in the medium tends to inhibit the growth of Rev. 1 more than that of other strains of *B. melitensis* but, if plates with dye are incubated in an atmosphere containing an added 10% of carbon dioxide, this difference is not so apparent.

3. Rev. 1 will not grow on agar containing 5 IU of penicillin per millilitre. Other strains of *B. melitensis* will grow under these conditions.

4. Rev. 1 will grow on agar containing 2.5 μg per millilitre of streptomycin, a concentration sufficient to inhibit the growth of other strains of *B. melitensis*.

**The use of Rev. 1 vaccine in the field**

The use of full doses ($1-2 \times 10^8$ organisms) of Rev. 1 should be confined to sheep and goats aged 3–8 months; that is, before there is any possibility of inadvertently vaccinating a pregnant animal. Vaccinated animals should not be used for breeding for at least 1 month after vaccination. The vaccine is administered subcutaneously. On no account should pregnant animals be vaccinated. Revaccination is not recommended. Dried vaccine should be used within 2 hours of reconstitution. Every effort should be made to keep liquid or reconstituted dried vaccine cool during use in the field.

Adult goats may be vaccinated with $10^4$ organisms; this dose may be given without danger of producing abortion in pregnant goats or excretion in lactating goats, it has the additional advantage of producing only a minimal serological response.
CHAPTER 5

BRUCELLA OVIS

Introduction

*Brucella ovis* is one of the etiological agents of epididymitis in rams. No one method of diagnosis is reliable in individual animals and best results are obtained when clinical examination, serology, and culture are used in combination. Diagnosis by clinical examination is an inadequate procedure, as other etiological agents cause clinically similar forms of epididymitis. In addition, some rams infected with *B. ovis* do not develop clinical epididymitis. When clinical epididymitis exists in a flock, serological procedures are adequate for determining that the condition is caused by *B. ovis*, but some infected rams may be serologically negative. Isolation and identification of the organism is the only procedure not subject to equivocation; however, *B. ovis* cannot be isolated from the semen of all infected rams.

Vaccination

An alum-precipitated bacterin \(^1\) is used for vaccination, but it gives rise to persistent vaccination titres that complicate the interpretation of serological results and limit diagnosis largely to clinical examination and isolation of the organism. *B. abortus* strain 19 vaccine has been used concomitantly with 1 dose of bacterin (Buddle, 1958) but occasionally strain 19 becomes localized in the testes and its use in sheep is prohibited in some countries. *B. melitensis* strain Rev. 1 has been used successfully in preventing *B. ovis* infection in rams (Van Heerden, 1964) and its use does not complicate subsequent serological investigations, but it is not recommended for use in areas where *B. melitensis* is absent. Control by vaccination is more satisfactory when clinical examinations are conducted annually and all rams with clinical epididymitis are removed from the flock.

Diagnostic procedures

Clinical examination

Clinically the disease is diagnosed by palpation of the epididymis. Both testicles are grasped from the rear of the animal and palpated simultaneously and a comparison made of the symmetry of size, shape, and consistency. The affected epididymis is slightly to greatly enlarged and is firm to quite hard, depending on the age of the lesion and the amount of fibrous tissue present. Most frequently the tail of one epididymis is affected, less frequently

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\(^1\) Ramedol-Cutter Laboratories, Berkeley, Calif., USA.
the head of one epididymis and least frequently the epididymis of both testicles. The cleft that separates the tail or head of the epididymis from the testicle is frequently obliterated and sometimes there is atrophy of the testicle.

Serological procedures

A rough specific antigen, of polysaccharide nature, extracted by heating saline suspensions of *B. ovis*, was shown to be the antigen that detects *B. ovis* infection by both the complement fixation and gel-diffusion tests (Myers et al., 1972). It is prepared as follows:

*B. ovis* is grown on Tryptose agar or other suitable medium, with 5% serum added, in Roux bottles. Incubation is for 3–5 days at 37°C in an atmosphere containing an added 10% carbon dioxide. The growth is harvested in normal saline solution, 10 ml per Roux bottle. The cells are washed 3 times in suspending fluid by centrifugation at approximately 10 000 g for 20 minutes. Following the third washing, the cells are resuspended in normal saline to the original volume, autoclaved at 120°C for 20 minutes, and when cool are deposited by centrifugation (10 000 g for 20 minutes); the supernatant is then removed for antigen. The antigen is passed through a bacteriological filter, then dialysed against cold distilled water for at least 48 hours to remove the anti-complementary activity, after which it may be freeze-dried or stored frozen in aliquots.

The complement fixation test

Both the methods given in Chapter 2, the composite method (pages 91–104) and Hill’s method (pages 104–110), are suitable for use in the *B. ovis* complement fixation test. The rough specific antigen is standardized as described on pages 98 and 99 for the composite method and page 107 for Hill’s method. An anti-*B. ovis* serum of moderate titre is used as standard. Liquid *B. ovis* antigen is diluted as indicated in Table 13 or 16. Freeze-dried antigen is reconstituted at the rate of 1 mg in 1 ml of distilled water, then diluted in the same way as the liquid antigen. In each method, the antigen dilution giving the highest titre with the standard serum is chosen for use in the diagnostic test. The antigen should not have any anti-complementary activity; if such activity is evident in the titration, further dialysis of the antigen is required. With Hill’s method the sera to be tested should be diluted 1:4 rather than 1:2.

Definite fixation at 1:40 should be regarded as positive, and at 1:20 as suspicious.

The gel-diffusion test

**Preparation of the gel medium**

Borate buffer 0.03 mol/litre pH 8.3

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>boric acid (H₃BO₃)</td>
<td>1.86 g</td>
</tr>
<tr>
<td>potassium chloride (KCl)</td>
<td>7.25 g</td>
</tr>
<tr>
<td>distilled water</td>
<td>950 ml</td>
</tr>
</tbody>
</table>
Dissolve the constituents, then adjust the pH to 8.3 with 0.2 mol/litre sodium hydroxide. Make up to 1 litre with distilled water.

**Gel medium**

- Agarose: 0.8 g
- Borate buffer 0.03 mol/litre pH 8.3 (above): 5 ml
- Saline solution (5% for sheep and goat sera): 93 ml
- 0.85% for other species: 93 ml

Steam to dissolve. Add 1 ml of 1% sodium azide and mix. Dispense the gel in plastic Petri dishes to a depth of 3–4 mm. When the gel has set, the wells are cut. A standard pattern of 6 wells around a central well, each 4 mm in diameter and 5 mm apart, is convenient. The test can be adapted to slides and other patterns.

**Optimum antigen concentration for the gel-diffusion test.** Reconstitute 5 mg of freeze-dried antigen in 1 ml of water and make 5 twofold dilutions in saline. If the antigen is in liquid form make similar dilutions. Using capillary or Pasteur pipettes place a known positive serum from a sheep naturally infected with *B. ovis* in the central well of one gel pattern and a positive serum from a sheep or goat naturally infected with *B. melitensis* in the central well of a second gel pattern. Place the dilutions of antigen in the surrounding wells of the two patterns. Put the Petri dishes, or slides, in a moist chamber (any closed container with soaked cotton pad will suffice) and examine after 24 hours at room temperature. The antigen concentration giving the clearest line or lines with *B. ovis* serum and no lines with the *B. melitensis* serum is the dilution used in the diagnostic test.

**The gel-diffusion test procedure.** Fill the central well with antigen diluted to its optimum concentration. Place undiluted samples of the test sera in the surrounding wells. A known positive serum should be included each day. Allow the tests to incubate in a moist chamber at room temperature. Precipitin lines usually develop within 24 hours but the test should be examined also after 2 and 3 days.

**Culture—Isolation and identification**

Isolation and identification of the organism is the only diagnostic procedure not subject to equivocation; however, negative culture results on semen are not sufficient evidence to rule out *B. ovis* infection. In the case of some chronically infected animals the organism may be shed intermittently in the semen or not at all. Difficulties may also be encountered in obtaining semen samples not contaminated with organisms that overgrow *B. ovis*.

The selective medium employed successfully for the isolation of other *Brucella* species (pages 17 and 18) inhibits the growth of *B. ovis*. The recent
development of selective media for the isolation of *B. ovis* (Brown et al., 1971) has made the isolation of *B. ovis* a practical technique for routine use (see Fig. 22).

**FIG. 22. CULTURE OF RAM SEMEN ON SELECTIVE MEDIUM FOR THE CULTURE OF B. OVIS**

Left—Serum Tryptose agar  
Right—Modified Thayer-Martin medium with nitrofurantoin

*Preparation of medium (modified Thayer-Martin medium with nitrofurantoin)*

Dissolve 36 g of GC Medium Base ¹ in 600 ml of distilled water. Dissolve 10 g of haemoglobin ² in 400 ml of distilled water. Sterilize each by autoclaving for 15 minutes and then cool each to about 57 °C. Dissolve the contents of a 10-ml vial of VCN ³ inhibitor in 10 ml of sterile distilled water. Dissolve 10 mg of sodium nitrofurantoin ⁴ in 1 ml of sterile distilled water or 10 mg of nitrofurantoin ⁴ in 1 ml of acetone. Combine all ingredients and mix thoroughly while keeping the temperature at 57 °C, preferably by means of a heating magnetic stirrer. Dispense into Petri plates.

*Collection of semen samples*

Restrain rams in lateral recumbency, clip hair and wool from around preputial opening and obtain semen by electro-ejaculation. As erection

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¹ Available from BBL, Division of BioQuest, Cockeysville, Md., USA.
² Contains 300 g of vancomycin, 7500 g of cetatin, and 12,500 IU of nystatin and is available from BBL Division of BioQuest, Cockeysville, Md. 21030, USA, or B-D Merieux, 69, Marcy l’Etoile, France.
³ Available from Easton Laboratories, Norwich, N.Y., USA.
⁴ Available from Serva, D-6900 Heidelberg, Federal Republic of Germany.
occurs direct the glans of the penis into a sterile Whirl-pak\(^1\) plastic bag or other suitable container in which the semen is collected.

The specimens are stored under refrigeration until cultured.

*Inoculation, incubation, and examination of the medium*

A sterile cotton swab is charged with semen and streaked backwards and forwards across the entire surface of the medium. The inoculated plates are incubated at 37° C for 4–7 days in a container with 5–10% carbon dioxide added. The plates are examined for growth under a low-power dissecting microscope with an incident light source. Colonies of *B. ovis* are round with an entire, even margin, and are convex with a smooth glistening surface. The centre of a colony is opaque and the periphery translucent. The colonies are indistinguishable in appearance from those of smooth species of *Brucella*.

Colonies are selected, characterized, and identified as described in Chapter 1.

\(^1\) Available from Nasco Industries Inc., Fort Atkinson, Wisc., USA.
CHAPTER 6

BRUCELLA CANIS

Introduction

*Brucella canis* was isolated and identified as an etiological agent of contagious abortion in dogs by Carmichael (1967). Since then the disease has been reported in at least 38 states of the United States; however, proof of infection has been based primarily on serological evidence.

The dog is the only animal species known to be naturally infected with *B. canis*. In addition to causing abortion in the female, it produces epididymitis, testicular atrophy, and occasionally sterility in the male. *Brucella canis* is also of importance from the public health standpoint since human infections have occurred as a result of laboratory accidents and of contact with infected dogs.

The disease is especially prevalent in breeding colonies that have a high rate of turnover in dogs. The results of vaccination and treatment studies have been unsatisfactory; therefore, control efforts should be directed toward identifying infected dogs by serological and bacteriological procedures and eliminating them.

Diagnostic procedures

Serological procedures

A unique characteristic of *B. canis* is that it is mucoid in nature and does not share the surface antigens of smooth brucellae. Therefore, the antigen used in serological testing for *B. abortus*, *B. suis*, and *B. melitensis* will not detect antibodies against *B. canis*. In addition, the mucoid property of the organism has complicated the development of a satisfactory stable antigen and an agglutinating test procedure as an aid in diagnosing the disease. Another complicating factor in serological testing is the occurrence of agglutinating reactions in sera from some dogs proved not to have been infected with *B. canis*.

The tests that are described in this chapter have given satisfactory results in detecting infected dogs. In addition, they have been effective in eliminating most reactions in sera from dogs proved not to have been infected with *B. canis*.

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1 A plate agglutination test has recently been described by George & Carmichael (1974).
The modified mercaptoethanol tube agglutination test

Preparation of stock reagents

(a) Stock solution of formalized saline. Add 10 ml of formaldehyde solution (37-40%) to 90 ml of 0.85% sodium chloride solution.

(b) Stock solution of sodium chloride (3.5% w/v). Add 3.5 g of sodium chloride to 100 ml distilled water.

(c) Sørenson’s phosphate-buffered saline (pH 7.0). Prepare as described on page 20.

(d) Stock antigen. The agar media used for other Brucella species (B. abortus, B. suis, B. melitensis), as described on pages 14-16, are satisfactory for antigen production. Agar slopes (30×200 mm) are inoculated with B. canis and incubated (without added carbon dioxide) at 37°C for 24 hours. The growth is washed from the surface of each slope with 7 ml of sterile 0.85% saline solution and used to inoculate a Roux flask of agar medium. Each inoculated Roux flask is rotated to assure even distribution of inoculum over the entire agar surface and is incubated in an inverted position at 37°C for 24 hours. The growth is washed from the surface of the agar of each Roux flask with 20 ml of Sørenson’s phosphate-buffered saline containing 0.06% of formalin, which is prepared by adding 0.6 ml of the stock solution of formalized saline to 99.4 ml of Sørenson’s phosphate-buffered saline. The harvests from the Roux flasks are pooled, filtered through several layers of sterile gauze and inactivated by heating to 70°C for 1 hour. After cooling, the suspension is centrifuged at 10,000 g for 30 minutes. The supernatant is discarded and the pellet is resuspended in Sørenson’s phosphate-buffered saline containing 0.5% of formalin, which is prepared by adding 5.0 ml of stock solution of formalized saline to 95.0 ml of Sørenson’s phosphate-buffered saline. The antigen is standardized to a density of 4.5% cells by using the packed cell method as described on page 61. The stock antigen is bottled as described on page 72 and stored at 4°C until used.

Antigen can also be prepared with B. canis propagated in a liquid medium by the batch method (pages 67-70) except that the Brucella cells are killed before harvesting by raising the fermentor temperature, after 24 hours of incubation, to 70°C for 1 1/2 hours.

Preparation of working reagents

(a) Working antigen (3.5% sodium chloride, 0.06% formalin, 0.2% cells). Prepare the diluent by adding 0.6 ml of the stock solution of formalized saline to 99.4 ml of the stock solution of sodium chloride. Add 4.4 ml of stock antigen to 95.6 ml of this diluent. Store at 4°C.

(b) Test diluent (3.5% sodium chloride, 0.06% formalin, 0.1 mol/litre 2-mercaptoethanol). Prepare by adding 0.6 ml of the stock solution of for-
malized saline to 99.4 ml of the stock solution of sodium chloride and then add 0.7 ml of 2-mercaptoethanol to 99.3 ml of this solution. Store at 4°C and prepare fresh every 2–3 weeks.

**Test procedure**

The test is set up using the decimal dilution method (page 72). However, only the 1:50 (0.04 ml of serum), 1:100 (0.02 ml of serum) and 1:200 (0.01 ml of serum) dilutions are used.

After measuring the sera into each tube, 1 ml of test diluent and 1 ml of working antigen are added. Mix by shaking and incubate at 37°C C for 48±3 hours. The tubes are observed in the same way as for the tube agglutination test (USDA), as described on page 74, except that the tubes are not shaken and reading is accomplished by determining the degree of clearing in the supernatant. The reaction is classified as negative, incomplete, or complete at the appropriate dilution.

**Interpretation**

(a) Negative—negative reaction at the 1:50 dilution.

(b) Suspicious—incomplete or complete reactions at the 1:50 or 1:100 dilutions or incomplete reaction at the 1:200 dilution.

(c) Positive—complete reactions at the 1:200 dilution.

A suspect animal should be retested in 30-60 days. If the titre is stable or decreasing, the animal is probably not actively infected with *B. canis*.

**The complement fixation test**

Hill's complement fixation technique (page 104) can be adapted for the diagnosis of *B. canis*. Antigen is prepared from a suspension of *B. canis* by the method described for the production of *B. ovis* antigen on page 145. It is necessary to titrate the antigen as described on page 107 for both anti-complementary activity and sensitivity.

The test is conducted with serum diluted 1:20 rather than serum diluted 1:2. A 1:20 dilution of serum is made with barbital-buffered saline solution (page 88) and inactivated by heating in a water bath at 58°C C for 30 minutes.

**Interpretation**

(a) Negative—negative reaction in first dilution (0.2 ml of 1:20 diluted serum).

(b) Suspicious—any reaction (≥ +) in the first dilution (0.2 ml of 1:20 diluted serum) or in the second dilution (0.1 ml of 1:20 diluted serum).

(c) Positive—any reaction (≥ −) in the third dilution (0.04 ml of 1:20 diluted serum) or in the fourth dilution (0.02 ml of 1:20 diluted serum).
A suspect animal should be retested in 30-60 days. If the titre is stable or decreasing, the animal is probably not actively infected with *B. canis*.

The gel-diffusion test

The gel-diffusion method, described by Myers & Siniuk (1970) can be adapted for the diagnosis of *B. canis* infection. The gel medium is prepared as described on pages 145–146 except that 0.85% saline, rather than 5% saline, is used. The antigen is prepared as described on page 145 and the optimum antigen concentration is determined as described on page 146, except that a known positive serum from a dog naturally infected with *B. canis* is used in the central well of one pattern. The test is conducted and interpreted as described for *B. ovis* on page 146.

It should be noted that *B. canis* or *B. ovis* can be used for preparing the antigen as cross-reactivity has been demonstrated (Myers et al., 1972).

Culture, isolation, and identification

Isolation and identification is the only method of establishing a definitive diagnosis. *Brucella canis* has been isolated from vaginal discharges, aborted fetuses, milk, urine, semen, and various tissues. However, blood is the bacteriological specimen of choice as infected dogs are bacteraemic before a diagnostically significant titre is detected and usually remain bacteraemic for long periods of time (up to several years). However, infected dogs remain serologically positive for several months after becoming abacteraemic.

Media

(a) Agar. The agar media used for isolating other brucellae (*B. abortus, B. suis, B. melitensis*), as described on pages 14-17, are satisfactory for isolating *B. canis*. A medium made selective by the addition of cycloheximide, bacitracin, and polymyxin B (pages 17 and 18) is recommended for potentially contaminated material (specimens other than blood) and for subculturing from broth.

(b) Broth. The media used for isolating other brucellae (*B. abortus, B. suis, B. melitensis*) as described on pages 14-17 are satisfactory. However, agar is not added and the broth medium is dispensed into culture tubes in 10-ml quantities.

Collection of specimens

Culture specimens, other than blood, are collected using procedures similar to those described for other *Brucella* species (pages 22–25). About 8 ml of blood is collected from the jugular vein with a syringe; 3 ml is put into a Vacutainer containing heparin (EDTA is inhibitory) and the remaining 5 ml (without anticoagulant) is put into a culture tube containing 10 ml of broth and is mixed, and then frozen at −20°C.
Inoculation, incubation, and examination

Two methods are used for culturing blood. The direct method provides results in a shorter time (in 4–7 days), but the method of subculturing from broth is more sensitive.

(a) Direct culturing of blood. With a sterile 1.0-ml pipette, deliver 0.1 ml of heparinized blood on to each agar plate and spread with a spreader. Incubate at 37°C (without added carbon dioxide) for 4–7 days. The plates are examined by obliquely reflected light as described on page 37. When a colony is touched with a needle or loop, it tends to stick to it. Colonies are selected, characterized, and identified as described in Chapter 1.

(b) Subculturing from broth. After the 10 ml of broth containing 5 ml of blood has been frozen at —20°C overnight, it is incubated at 37°C (without added carbon dioxide) for a minimum of 6 days. If B. canis was not isolated by direct culturing of blood, 0.1 ml of blood–broth mixture is inoculated on to plates of agar medium made selective by the addition of cycloheximide, bacitracin, and polymyxin B. The inoculated plates are incubated and examined as described for the direct culturing of blood.

Colonies are selected, characterized, and identified as described in Chapter 1.
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