Chapter 4

BRUCELLOSIS

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4.1 SUMMARY

Brucellosis in sheep, caused by *Brucella melitensis*, is essentially an abortive illness of the ewe. It is highly contagious and hazardous to man. Direct diagnosis involves the isolation of the bacteria on a selectively adapted medium, followed by identification by phenotyping tests (lysotyping, biochemical and serotyping) or by molecular methods (PCR-RFLP). Indirect diagnosis is undertaken using serological tests (Rose Bengal and complement fixation) and allergen tests (delayed hypersensitivity to *Brucella*).

4.2 INTRODUCTION

Brucellosis, caused by *Brucella melitensis*, is a contagious infection which affects the reticulo-endothelial systems and reproductive organs of small ruminants. *Brucella melitensis* has a predilection for the pregnant uterus and the mammary gland. The main clinical manifestation is abortion in ewes and goats and less commonly epididymitis in rams and billy-goats. The cyclic aspect of abortive storms, interrupted by a short or long silent phase, is characteristic of the development of *B.melitensis* infection within a flock.

The products of a past infection or the infected material associated with them are nearly always the source of transmission. Excretion of bacteria in milk is a danger to humans but does not appear to be a source of inter-animal infection except perhaps via the hands of the milker. The most common route of infection is via the naso-pharynx but entry through the skin is possible particularly through abrasions. Having crossed the mucus or cutaneous barrier, the *Brucella* passes via the lymphatic system to the nearest lymph nodes, usually those of the head. In the resistant animal, the bacteria are killed by macrophages, cellular components of the immune system, aided by certain antibodies and activated by T lymphocytes. In the susceptible animal, on the other hand, the bacteria multiply in phagocytic cells, kill and lyse them and go on to infect new cells. Bacteraemia results although *Brucella* rarely occur in large numbers in the general circulation. If the ewe or goat is pregnant, the bacteria usually invade the uterus and multiply in the placenta. Eventually they infect the foetus and cause a partial infection, with or without abortion, which releases large quantities of *Brucella* into the environment. During chronic infection certain lymph nodes are automatically infected whether or not the animal is pregnant. The mammary gland is a common target organ and provides a favourable environment for the persistence of infection and *B.melitensis* excretion in the milk. In the male, the infection preferentially affects the genital system - the epididymis, testicles, seminal vesicles - with frequent excretion of the bacteria in the sperm and occasionally clinical expression as epididymitis.

Brucellosis in small ruminants is controlled by prophylaxis and/or medication. Prophylactic methods alone, based

on the detection and slaughter of animals which react positively in immunological tests, is only effective in regions where the prevalence of the infection is low and contagion can be well controlled. The method is ineffective in regions of high infection where positive animals must first be treated by a preliminary phase of vaccination. This reduces the prevalence rate to an acceptable level to allow the introduction of a combined approach of vaccination associated with culling of infected animals (medical-sanitary). Finally, vaccination is omitted and any infected animals are identified and culled until the disease is eradicated.

The live vaccine, Rev. 1, is produced by a laboratory induced mutation of *B.melitensis* and has been the basis of the anti-Brucella vaccine for sheep and goats for many years. The vaccine is normally administered subcutaneously at a dose rate of 1 x 10^9 viable bacteria. Under these conditions a serological response is induced which lasts longer in older animals than in young ones. This limits the vaccines usefulness in young animals, although antibodies do persist until an adult age in 1-2% of young animals. The procedure of conjunctival administration [5, 6], which limits the spread of the vaccinal bacteria to the lymph nodes in the cervical region, induces a solid immunity whilst limiting the serological reaction to a period usually no longer than four months. This process therefore renders the use of the Rev 1 vaccine compatible with prophylactic sanitary measures and allows quicker eradication of the infection.

The infectious disease, known as *contagious ram epididymitis*, involves another aetiological agent, *Brucella ovis*, a naturally "rough" species with exclusive pathogenicity for the sheep under natural conditions. Ewes are sensitive to *B.ovis* but infection is usually inapparent and very rarely results in abortions or foetal deaths. In the latter case it is difficult to differentiate between *B.ovis* and occasional "rough" varieties of *B.melitensis*.

4.3 SAMPLES

The samples recommended for bacteriological examination, the optimal sampling time and the conditions of transportation

Table 4.1 : Recommended samples for bacteriological examination of the aborted female

	Recommended samples	**Sampling time**	**Transport and storage conditions**
Live animal	- Vaginal swab - milk (about 20 ml for each quarter)	Preferably during the 15 days after birth or abortion	All samples should be packaged in waterproof containers and maintained at 4°C if analysis is undertaken winthin 48 hours or at -20°C if analysed later. For transportation it is best to use insulated packaging, conform to safety standards and keep journey times to a minimum
Dead animal	- lymph nodes (retropharyngeal,sub maxillary, retromammary - uterus - liver - mammary gland	From the carcass at the autopsy	
Aborted fœtus	- stomach fluid - liver - lung	As soon as possible after the abortion	

are summarised in Table 4.1. Samples must be collected as aseptically as possible. For milk samples, the teats must be disinfected and dried, the first two milk jets discarded and the following milk collected in a sterile container avoiding any contact with the hands. Table 4.1 does not include samples to be taken from the male since these are not of direct concern in the present context (if necessary, see Alton et al [1]).

Blood samples for serological examination should be collected in silicone coated, sterile tubes (e.g. Vacutainers) and removed to the laboratory as quickly as possible.

4.3.1 Treatment of samples for bacteriological examination

Whatever the sample type, bacterial multiplication in a selective medium will increase the chances of isolating *Brucella.*

4.3.1.1 Milk

1. Centrifuge approximately 10ml of milk, taken from the two quarters of the udder and mixed together, at 3000 - 5000 rpm for 15 minutes.
2. Spread the cream over half of the surface of a plate selective medium.
3. Empty the intermediate liquid into a receptacle containing disinfectant, spread the precipitate over the other half of the medium and incubate the plates at 37°C.

4.3.1.2 Vaginal swabs

Remove the swab from its protective sheaf, smear the sample onto the surface of a plate of selective medium and incubate the plates at 37°C.

4.3.1.3 Tissues (organs and lymph nodes)

Any system of maceration may be used but a "stomacher" (Seward Medical, London, UK) which permits disruption of the tissue in watertight plastic bags is especially recommended.

1. Dip the sample rapidly into 95% ethanol, flaming as a precaution, and place in a sterile Petri dish then cut up the sample.
2. Trim the tissue if necessary and chop coarsely using scissors.
3. Grind the tissue.
4. Take a sample of the ground tissue using a flamed and cooled spatula and spread over the surface of the selective medium. Incubate the plates at 37°C.

4.3.1.4 Stomach fluid

Stomach fluid is the best sample to take from the aborted foetus. Remove a sample using a platinum loop or a syringe and spread over the surface of the selective media.

4.4 RISKS TO HUMAN HEALTH

Brucella is classified in risk group III [2], which necessitates strict protective measures during handling: working in class L3 containment areas or, if unavailable, in a conventional laboratory equipped with a laminar flow hood. In all circumstances it is essential to use good bacteriological practices, namely:

- Wear gloves during handling of samples.
- Never pipette directly by mouth.
- Avoid producing aerosols.
- Disinfect the work surfaces after all manipulations.
- Decontaminate re-useable materials by autoclaving, and incinerate waste.
- Wash hands with a disinfectant soap.
- For more details refer to the OMS Manual [2] or to the paper of Alton et al [1].

4.5 DIRECT DIAGNOSIS

Direct diagnosis of brucellosis is achieved by isolation and identification of the responsible micro-organism using bacteriological tests which determine the phenotypic characteristics of the bacteria. *Brucella* can also be detected using molecular tests which take account of all the characteristics of the genome.

4.5.1 Bacterial detection

4.5.1.1 Bacteriological

Principle

This technique consists of demonstrating the presence of the bacteria in smears or histological sections of tissues by specific staining. The Stamp stain is the classical method used for the bacteriological identification of *Brucella*.

Materials and Reagents

- Staining boat.
- Immersion microscope.
- Ziehl Fuchsin stain (stock solution: dissolve 1g of basic fuchsin in 10ml of absolute alcohol and add 90ml of 5% phenol solution).
- 0.5% acetic acid.
- 1% methylene blue.

Procedure

1. Take a smear or impression of sample and fix by flaming.
2. Stain with Ziehl Fuchsin diluted 1 in 10, for 10 minutes.
3. Wash under running water.
4. Destain with 0.5% acetic acid for a maximum of 30 seconds.
5. Wash under running water.
6. Counterstain with a 1% methylene blue solution for 20 seconds.
7. Wash under running water and dry.

Reading and interpretation of results

Brucella appear as red bodies on a blue background, isolated or in groups, usually intracellularly. A positive result is not necessarily confirmation of infection. This stain is subject to errors due to excess or lack of organisms and the result, whether positive or negative, must always be confirmed by culture.

4.5.1.2 Bacterial isolation

a / By selective media

The selective medium recommended for the isolation of *Brucella* is Farrell's medium [4] prepared as follows :

- To one litre of basal media BAB (Blood-Agar-Base No 2, Oxoid) or BMB (Brucella-Medium-Base, Oxoid), cooled to 50°C, add 5% sterile horse serum plus the following antibiotics :

- cyclohexamide	100 mg
- bacitracin	25000 units
- polymixin B sulphate	5000 units
- vancomycin	20 mg
- nalidixic acid	5 mg
- nystatin	100000 units

- Pour into Petri dishes.
- Incubate overnight at 37°C then store at 4°C. Under these conditions the dishes are stable for up to one month.
- Oxoid retail the antibiotic supplement (ref. SR 83) ready prepared in flasks containing the necessary quantities for 500ml of base media.

b / By inoculation in laboratory animals

Farrell's medium provides an efficient method of isolation and it is rarely necessary to resort to inoculation of guinea-pigs or mice except in cases where the sample is highly contaminated (for more details consult Alton et al [1]).

4.5.2 Identification of the *Brucella* genus

On Farrell's medium, colonies of *Brucella* are visible after 2 to 3 days incubation. After five days the colonies are translucent, convex, light blue, with regular edges and a diameter of 0.5-1.0mm. If the sample is heavily infected a dark blue coating of culture is apparent after 48 hours.

4.5.2.1 Microscopic examination

Brucella are small coccobacillus organisms (0.6-1.5µm x 0.5-0.7µm), immobile, Gram-negative, usually occurring singly but sometimes in pairs or groups. They do not have a cell wall and do not show bipolar staining.

4.5.2.2 Simple bacteriological tests for identification of the Brucella genus

Positive Tests	Negative Tests
- Strict aerobes	- Haemolysis
- Catalase	- Acidification of glucose
- Oxydase *	- Indole
- Reduction of nitrates to nitrites*	- Citrate
- Urease*	- Methyl red and Voges Proskauer

** B. ovis gives negative results to these tests.*

The urease test for Brucella is best undertaken on Christensen medium which has the following composition :

- Peptone 1 g
- Sodium chloride (NaCl) 5 g
- Potassium phosphate (KH_2PO_4) 2 g
- Phenol red 0.0012 g
- Dextrose 1 g
- Agar 20 g
- Distilled water 1 litre
- Adjust to pH 6.8 and aliquot prior to sterilisation.
- Add urea (20% solution sterilised by filtration) to a final concentration of 2% before pouring the medium into Petri dishes. For effective growth cultures seeded onto Christensens medium should come from a solid culture. A positive reaction is indicated by a change in the colour of the medium around the edge of the culture from yellow to a fairly bright red, the rate of production depending on the Brucella strain (1 to 15 minutes).

4.5.2.3 Control of the purity and morphological typing of colonies

Colonies suspected of being *Brucella* after the previous tests should be seeded onto plates of a non-selective base medium to confirm the purity of the strain and the morphological type of the colonies prior to continued identification.

Commercially available media based on Tryptic soya, solid (TSA) or liquid (TSB), are highly suitable for culture of *Brucella* : BBL Tryptose, Difco Bacto-tryptose, Gibco Tryptose agar, Oxoid Tryptose agar or bioMerieux Tryptose agar. 5% serum must be added for growth of *B.ovis*.

a / Colony observations without staining

Colonies should be observed by oblique transillumination using the apparatus shown in Figure 4.1. The apparatus comprises a light source (1) the rays from which converge on a plain mirror (3) and are returned, following a 45°

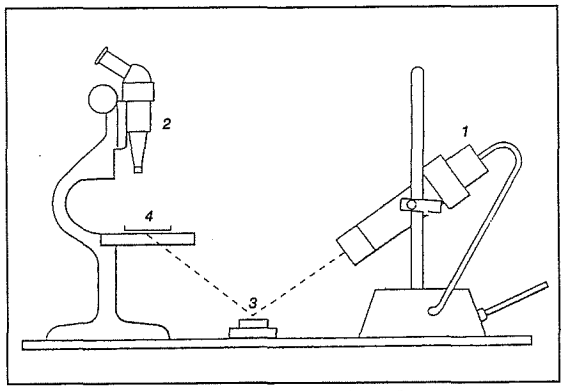

Figure 4.1 : Apparatus for observing colonies of Brucella

turn, to the plate placed on the support of the stereo microscope (2).

This set up allows the purity of the strain and the morphological type of the colonies to be assessed: smooth colonies are blue, iridescent and finely textured, rough colonies are more yellow and more granular in appearance (Figure 4.2, page 90).

b / Colony observations after staining with crystal violet

This staining method aids differentiation of the two morphological types, rough and smooth.

The stock solution of crystal violet is prepared as follows:

- dissolve :

• 2g of crystal violet in 20ml absolute alcohol;

• 0.8g of ammonium oxalate in 80ml distilled water;

- mix and store this stock solution for up to three months at 4°C.

- At time of use :

1. Dilute the solution 1 in 40 in distilled water.

2. Flood the plates to be examined and allow to react for 15-20 seconds.

3. Remove the surplus stain into a disinfectant with a Pasteur pipette and observe the plates by stereo microscope.

Smooth colonies will not be stained and will appear yellow on a violet background. Rough colonies will be stained violet-red (Figure 4.3, page 90).

Colonies of *B.melitensis* isolated from samples are usually of the smooth type whereas *B.ovis* is a naturally rough species.

Note

The Crystal violet does not affect the viability of the *Brucella* in the minutes following staining, allowing colonies to be subcultured to determine their type.

4.5.3 Determination of species

Differentiation of *B.melitensis* species within the *Brucella* genus is achieved by two classical tests: sensitivity to bacteriophages and determination of the metabolic oxidation profile by manometric methods.

4.5.3.1 Sensitivity to bacteriophages

A number of phages have been described in the literature. Three of them are particularly well adapted for differential diagnosis of *B.ovis* and *B.melitensis* :

• Tb phage, isolated by Tbilisi in the USSR, lytolytic for smooth *B.abortus* ;

• Iz phage, lytolytic for all species of smooth *Brucella* (therefore *B.melitensis*);

• R/C phage, lytolytic for most of the rough *Brucella*.

These bacteriophages, although not commercially available, can be obtained from the FAO/WHO Collaborating Centre for Reference and Research on Brucellosis, CVL, Weybridge, New Haw, Surrey KT15 3NB, UK. They are used in the test at a standard working concentration (SWC).

a / Determination of the SWC

Materials and reagents

- Tubes containing 4.5ml TSB media.

Procedure

1. Remove a 24 hour culture of the strain of *Brucella* under investigation from TSA agar and make a suspension (titre of approximately 10^9 cells per ml) in physiological saline.

2. Seed a tube of TSA agar by the appropriate technique (e.g. flooding, double coating) to obtain a homogeneous cover of culture and allow to dry for a few minutes.

3. For each run prepare a series of 10-fold dilution's of the test phage in TSB media.

4. Using a Pasteur or calibrated pipette, place a drop (20µl) of each dilution of the phage without touching onto the surface of the agar.

5. Once the drops have been absorbed, incubate the tubes at 37°C, with or without CO_2, according to the requirements of the strain.

Reading and interpretation of results

Read after 24 hours incubation or after 48 hours for strains that grow more slowly. The SWC is the highest dilution which gives a complete lysis (Figure 4.4, page 90).

When using a calibrated pipette, an estimation of the titre of the phage suspension can be made by counting the number of plaques of lysis at one of the lower dilutions and multiplying this by the dilution factor.

Note

• Handling of the phages must be undertaken with the greatest of care to avoid the formation of aerosols which may cause contamination of the *Brucella* cultures. The pipette must be held upright when depositing the drops to avoid spraying phages over the cultures.

• The diluted phages can be stored at 4°C for up to one month but the stock suspensions must be frozen.

b / Lysodiagnosis

The combined use of the three bacteriophages, at SWC, allows *B.melitensis* to be differentiated from the other smooth *Brucella* and from *B.ovis*.

Materials and reagents

- Sterile swabs.
- Plates of TSA agar plus 5% sterile horse serum.

Procedure

1. Remove a 24 hour culture of the strain to be tested from TSA agar and make up suspensions of similar concentrations in 0.5% sterile physiological saline.

2. Soak a sterile swab in each suspension and make streaks, one for each of the test phages, across the surface of the agar plates, without resoaking the swab (it is possible to make up to five separate streaks of culture on one plate). Allow to dry for a few minutes.

3. Using a Pasteur pipette, place a spot of the SWC of each phage in the middle of the streaks without touching the surface of the agar.

4. Once the spots have been absorbed incubate the dishes at 37°C, with or without CO_2, according to the conditions required for each strain.

Reading and interpretation of results

Read after 24 hours incubation or 48 hours for strains which grow more slowly. A positive result is indicated by the absence of culture around the position the phage was deposited.

B.melitensis is only lysed by the Iz phage and *B.ovis* by the R/C phage (Figure 4.5, page 90).

Note

The quality of interpretation of the test depends on:

- Inclusion of 5% serum in the base media, preventing the lypholytic activity of the R/C phage.
- The homogenicity of the morphological type, smooth or rough, of the strain of *Brucella* submitted for testing.
- The systematic inclusion of standard strains representative of the different lysotypic profiles.

4.5.3.2 Metabolic oxidation

This technique, based on the ability of suspensions of non-proliferating cells of Brucella to utilise oxygen in the presence of certain substrates (amino acids, sugars), allows determination of specific metabolic profiles relating to the morphological type of the strain (smooth or rough). This method, which is hazardous to perform, requires specific materials (Warburg apparatus) and highly competent personnel. The following laboratories are able to carry out this test on request, but samples must only be submitted by previous arrangement :

- FAO/WHO Collaborating Centre for Reference and Research on Brucellosis, CVL, Weybridge, New Haw, Surrey KT15 3NB, UK.
- United States Department of Agriculture, National Veterinary Service Laboratories, P.O. Box 844, Ames, IA, 50010, USA.
- INRA, Laboratoire des Brucella, Laboratoire de Pathologie Infectieuse et d'Immunologie, 37380 Nouzilly, France.

4.5.3.3 Molecular identification of B.melitensis and B.ovis

A number of tests using molecular biological techniques are proposed today for identification and typing of *Brucella*. One which distinguishes *B.ovis* and *B.melitensis* from other *Brucella* is presented here. The identification of bacteria by molecular biological techniques requires initial extraction of the genomic DNA.

a / DNA extraction

The proposed technique, adapted for *Brucella*, allows recovery of 100µl of good quality DNA containing approximately 1mg/ml.

Principle

The DNA is extracted in three main stages: (1) Lysis of the bacterial cells, (2) purification by a series of phenol-chloroform extraction cycles, (3) precipitation by frozen ethanol.

Materials and reagents

- Refrigerated centrifuge with rotors suitable for 10ml tubes and microtubes (Eppendorf).
- Desiccator or Speedvac.
- Electrophoresis apparatus.
- TENa buffer (50mM Tris, 50mM EDTA, 100mM NaCl, pH 8.0).
- 10% Sodium dodecyl sulphate (SDS).
- Proteinase K (20mg/ml) stored frozen at -20°C.
- 5M sodium perchlorate.
- TE buffer (10mM Tris, 1mM EDTA, pH 8.0).
- Agarose.
- Cold ethanol (frozen at -20°C).
- DNA marker of known molecular mass.

Procedure (Figure 4.6)

- Lysis of Bacterial Cells

1. Remove a 24 hour culture from the surface of TSA agar and suspend in 3ml sterile distilled water and centrifuge at 4500 rpm at 4°C for 10 minutes (in 10ml tubes).
2. Remove the supernatant and add 567µl of TENa buffer.
3. Resuspend the pellet (by vortex) and transfer to a microtube.
4. Add 30µl of SDS and 3µl of Proteinase K.
5. Mix gently by rotation of the tube and incubate for up to one hour at 37°C.

- Deproteination and Purification

6. Add 600µl of phenol and shake vigorously until a homogeneous white suspension is obtained.
7. Centrifuge at 13500 rpm at 4°C for 20 minutes.

8.Transfer 500µl of the upper aqueous phase into a new microtube.

9. Add 80µl of sodium perchlorate and *600µl of chloroform, shake.

10. Centrifuge at 5000 rpm at 4°C for 10 minutes.

11. Gently remove 500µl of the aqueous phase into a new microtube.

12. If necessary repeat from * until all trace of the interface disappears.

- Precipitation With Ethanol and Titration of DNA

13. Add 1ml cold ethanol and mix by gently turning the tube (filaments of DNA appear rapidly).

14. Centrifuge at 13500 rpm at 0°C for 10 minutes.

15. Remove the ethanol by turning the tube upside-down and leave to drain for a few seconds on absorbent paper.

16. Dry the precipitate by Speedvac for 5-10 minutes or in a disiccator.

17. Add 100µl of TE buffer to the precipitate and leave the DNA to re-enter the solution overnight at 37°C.

18. Determine the approximate concentration of DNA of an aliquot by electrophoresis on agarose gel in comparison with a marker of known molecular mass.

Note

- The first part of the extraction, up to the addition of phenol, must be undertaken using normal bacteriological precautions as described earlier. Phenol and chloroform must be handled under a hood.
- The DNA can be stored for up to one month at 4°C or frozen.

b / Restriction analysis of gene amplification product coding for the 25kDa Membrane protein

Molecular typing of these bacteria is based on the restriction analysis (RFLP) of the "Polymerase Chain Reaction" (PCR) amplification products of certain genes of *Brucella* [3]. The PCR-RFLP of the gene for the 25kDa outer membrane protein (OMP) of *Brucella* is particularly useful for the differential diagnosis of *B.melitensis* and *B.ovis*.

Principle

The aim of PCR is to exponentially amplify a specific DNA fragment. The different stages consist of denaturation at a high temperature of a fragment of double stranded DNA, hybridisation of each strand containing the region of interest which is to be amplified using a specific primer, and finally synthesis of the complementary sequence by DNA polymerisation. After n cycles the number of amplified fragments is 2^n.

Cleavage of the amplified product by an endonuclease allows production of restriction fragments of different lengths, correlated with the degree of polymorphism of the gene (RFLP).

Materials and reagents

- Thermocycler.
- Microtubes for PCR.
- Electrophoresis apparatus.
- UV light box (254nm).
- Agarose.
- Restriction endonuclease EcoRV.
- Molecular weight markers.
- DNA genome of *Brucella* + standard heterologous DNA.
- Primers:
  - 25A (5'-GGACCGCGCAAAACG-TAATT-3').
  - 25B (5'-ACCGGATGCCTGAAAT-CCTT-3').
- PCR kit including: Taq polymerase, dNTP, buffer ...

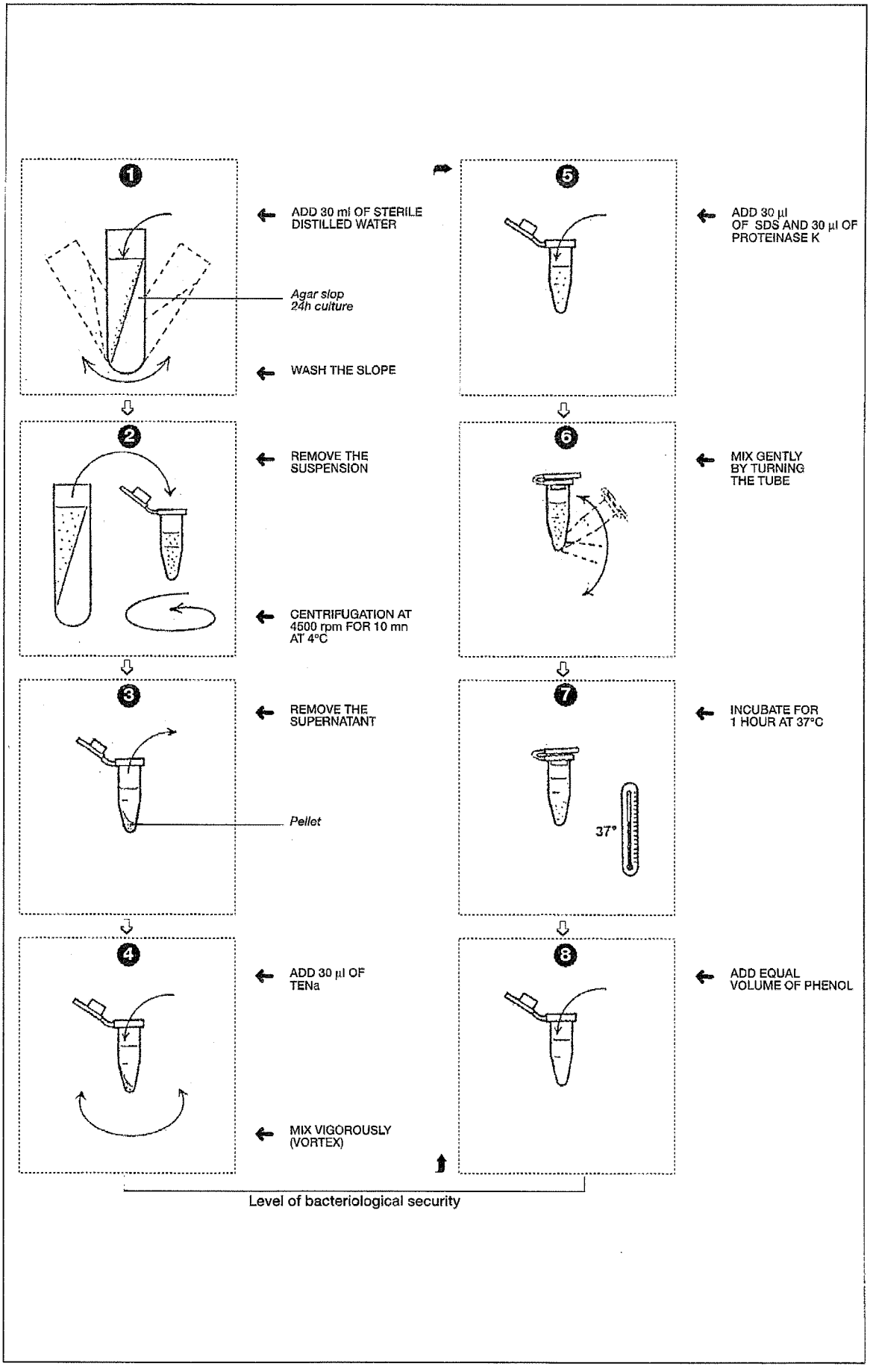

Figure 4.6 : Protocol for the extraction of Brucella DNA

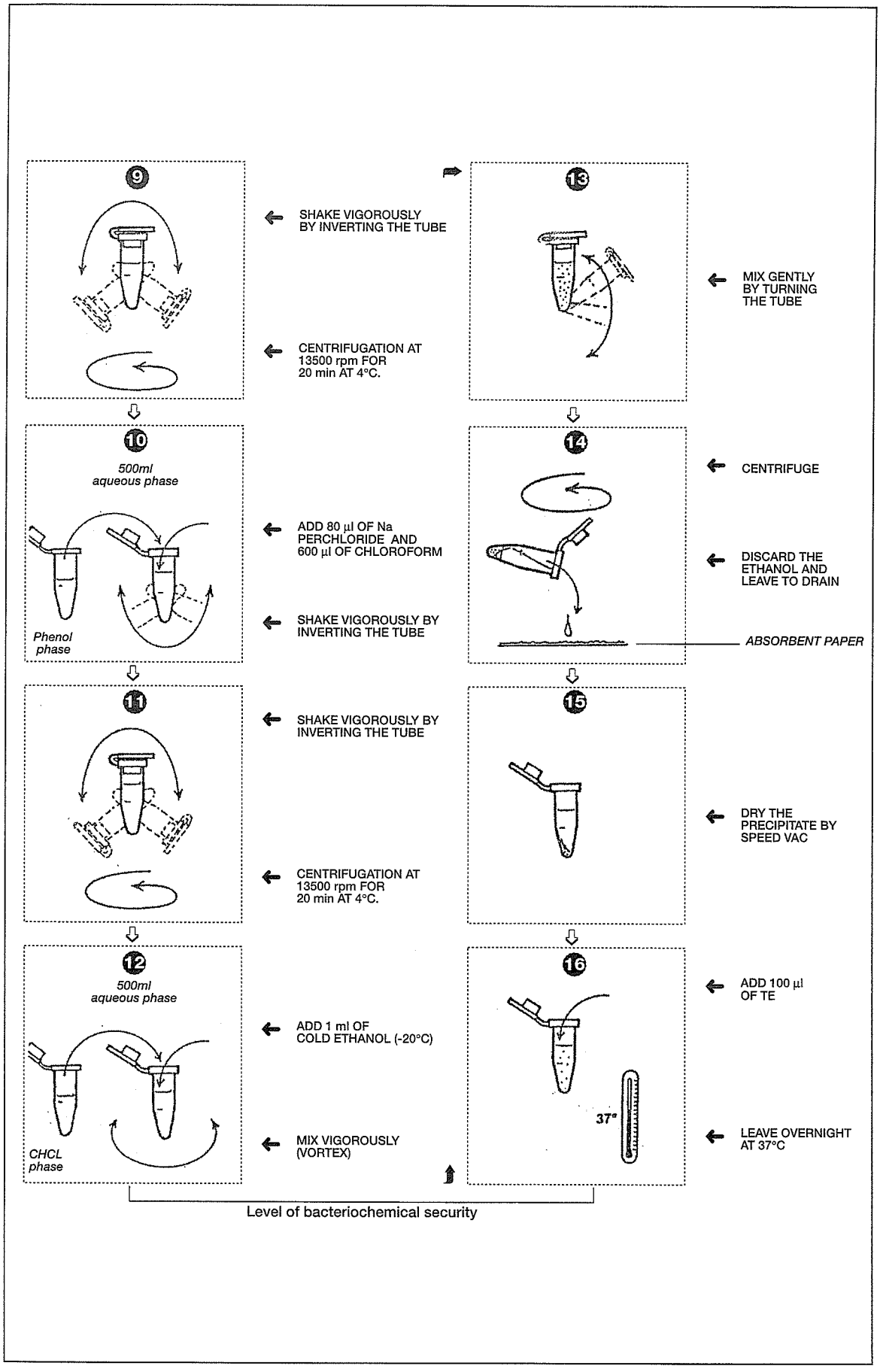

Figure 4.6 : (continued)

Procedure

The general conditions for PCR are adapted according to the type of thermocycler and the PCR kit used.

For an optimal amplification of the 25kDa OMP gene, under the specific conditions, the concentrations of reagents and the temperature are:

- for a total reaction volume of 100µl per tube :

-1µM of each primer;

- 100ng of *Brucella* genome DNA or standard heterogeneous DNA;

- 2.5 units of Taq polymerase;

- the temperature cycles for the denaturation, hybridisation and extension stages, respectively are 95°C, 58°C and 70°C although these temperatures can vary with the type of thermocycler used. The following programme is therefore given as an example:

- First cycle:	95°C for 5 min
	58°C for 2 min
	70°C for 3 min
- Following 30 cycles:	95°C for 1 min
	58°C for 2 min
	70°C for 3 min
- Final cycle:	95°C for 1 min
	58°C for 2 min
	70°C for 10 min

Approximately 5µl of each PCR product is subjected to electrophoresis on agarose gel in order to check the specificity and quality of amplification.

These products are then digested with EcoRV, following the method recommended by the supplier, and subjected to electrophoresis on agarose gel to allow assessment of the restriction profiles.

Reading and interpretation of results

The results are illustrated in Figure 4.7, page 91.

Migration of the PCR products on the agarose gel allows differentiation of *B.ovis* from other *Brucella* since it has the simplest profile as a result of the deletion of the 36 bp.

The results of restriction analysis confirm this observation and also show the absence of the EcoRV site for *B.melitensis*.

Note

• Precautions must be taken (location, materials) when carrying out this technique to avoid any contaminants that may introduce foreign DNA. It is therefore advisable to undertake each amplification alongside two standards :

- one tube containing all the reagents with the exception of the DNA (contamination control);

- one tube in which the *Brucella* DNA is replaced by a heterologous DNA (to control the specificity of the primers).

• Neither of these standards should show any traces of amplification in the zone of the 25kDa OMP gene.

4.5.4 Biotype determination

The differentiation of biotype of the six main species of *Brucella* is achieved by four tests: requirement for CO_2, production of H_2S, growth in the presence of stains and agglutination by monospecific sera.

4.5.4.1 Requirement for CO_2

This test determines the absolute requirement of the developing culture for CO_2. It must be carried out as soon as possible after isolation, at the time of transfer from selective medium to non-selective medium for purity control.

Procedure

1. Emulsify a few colonies of the strain to be tested in 0.5ml of sterile physiological saline.

2. Undertake isolation on four plates of TSA media.

3. Incubate two plates in an incubator at 37°C with normal atmosphere and the other two plates in an incubator at 37°C with the atmosphere enriched with 5-10% CO_2.

Reading and interpretation of results

B.melitensis strains do not require CO_2 for their growth and will therefore be present on all four plates. *B.ovis*, which requires CO_2, will only be present on the plates incubated in the presence of CO_2 and supplemented with 5% serum.

4.5.4.2 Production of H_2S

For *Brucella*, production of H_2S is determined using strips of paper impregnated with lead acetate.

Principle

When a strain produces hydrogen sulphide, the sulphur combines with the lead to form a black precipitate of lead acetate in the strips.

Materials and reagents

- Strips of paper impregnated with basic lead acetate (Prolab, code 26 488 261) prepared as follows:
  - Soak a sheet of filter paper in a 10% solution of lead acetate and leave to dry at room temperature.
  - Cut strips approximately 12 x 1 cm and store in the dark without sterilisation.

Procedure

1. Prepare a BAB gel slope.
2. Introduce a strip of lead acetate impregnated paper into the tube avoiding contact with the gel and secure the end of the strip between the bung and the side of the tube.
3. Incubate at 37°C, with or without CO_2 according to the requirements of the strain.

Reading and interpretation of results

Read after 24 hours incubation. The paper will remain white with strains of *B.melitensis* and *B.ovis* that do not produce H_2S.

Note

- It is recommended that BAB medium is used as opposed to TSA medium because the latter is likely to have a high concentration of sulphur containing amino acids which may give "false positives".

4.5.4.3 Growth in the presence of stains

The different Brucella types are sensitive or resistant to thionin or to basic Fuchsin incorporated directly into the base media.

Materials and reagents

- Sterile swabs ;
- BAB medium in 100ml flasks ;
- Staining solutions (0.1%) prepared as follows :
  - dissolve 0.1g of basic Fuchsin in 100ml distilled water
  - dissolve 0.1g of thionin in 100ml distilled water
  - sterilise these two solutions using a steamer or in a boiling water bath for one hour and store at 4°C for 3-4 months.

Procedure

1. Twenty-four hours before performing the test:
   - Remove samples of the test and reference strains from the surface of TSA agar and incubate at 37°C, with or without CO_2 according to the requirements of the strain.
   - Place 2ml of the made up staining-solutions (20µg/ml) and 98ml of BAB medium plus 5% horse serum in the stai-

ning plates, alongside a plate of the same base medium without staining solution. Leave overnight at 37°C.

2. On the following day, prepare the suspensions and seed the plates by streaking in the same way as for the lysotyping tests.

3. Incubate the dishes preferably in an atmosphere of 10% CO_2.

Reading and interpretation of results

Read after 3-4 days incubation. All strains of *B.melitensis,* whatever their biotype, will grow in the presence of the thionin and Fuchsin, but *B.ovis* will not grow in the presence of thionin (Figure 4.8 page 91).

Note

It is necessary to include during each test run:

- representative standards of the different growth profiles (media control);
- the seeding, under the same conditions, of the plate of medium without staining solutions (strain viability control).

4.5.4.4 Agglutination with monospecific sera

This test is used to determine the *B.melitensis* types. It is the only test that differentiates the three biotypes of this species.

Principle

All smooth *Brucella*, independent of species, possess two determinant surface antigens, A and M, distributed in different proportions according to the strain. Characterisation of the three possible antigen profiles lies in a simple plate agglutination test using specific sera of A and M antigens. A-dominant strains are only agglutinated by anti-A sera, M-dominant strains by anti-M sera and strains containing notable quantities of both epitopes by both sera. The methods of preparation of these sera are described by Alton et al [1] but it is also possible to obtain sera for comparison from the FAO/WHO Centre of Reference at Weybridge, mentioned previously.

Materials and reagents

- Glass or wooden rods.
- Monospecific anti-A and anti-M sera, tested against representative strains with the three surface antigen profiles and, if necessary, diluted in 5% phenol in physiological saline to give the agglutination patterns relating to these profiles. The working dilutions can be stored at 4°C for up to one month but must not be frozen.

Procedure

1. Place a spot of each of the diluted anti-A and anti-M sera on a slide in a Petri dish.

2. Using a loop, sample a few colonies of the test strain and place by the side of each spot.

3. With two different rods make a homogeneous bacterial suspension in each spot of serum.

4. Close the lid of the Petri dish and holding firmly gently rock to aid the mixing of the agglutinants.

Reading and interpretation of results

Only agglutinations appearing within one minute are considered as positive. The three biotypes of *B.melitensis* are uniquely distinguished on the basis of their agglutination: A-, M+ (biotype 1), A+, M- (biotype 2) and A+, M+ (biotype 3). B.ovis is not agglutinated by anti-A or anti-M sera but possesses an antigenic determinant common to all rough *Brucella.* A serum prepared against this determinant (see Alton et al [1]) agglutinates *B.ovis* but also rough varieties of *B. melitensis.*

Note

• All new batches of sera must be tested alongside representative strains of all three types of agglutination.

4.5.5 Differentiation between the Rev. 1 vaccinal strain and wild strains of *B.melitensis*

The Rev. 1 vaccinal strain is a modified biotype 1 strain of *B.melitensis*. In exceptional cases where *Brucella* is suspected of having caused abortion in vaccinated animals it is useful to be able to differentiate between wild strains of *B.melitensis* and the vaccinal strain. Differential diagnosis is basically achieved by a growth test in the presence of two antibiotics, penicillin (5 IU/ml) and streptomycin (2.5 µg/ml), on TSA media. The test cultures are prepared and seeded by streaking in the same way as for the lysotyping tests and for growth in the presence of stains. Results are read after 48 hours incubation. In contrast to biotype 1 wild strains of *B.melitensis*, the Rev 1 strain is sensitive to penicillin and resistant to streptomycin.

4.6 INDIRECT DIAGNOSIS : IMMUNOLOGICAL TESTS

In the live animal, bacteriological examination is not always practical. Diagnosis of brucellosis in individuals and groups of small ruminants is achieved by three main immunological tests: the Rose Bengal agglutination test, complement fixation and the allergen test.

4.6.1 Antigenic buffer test (Rose Bengal)

Principle

An antigenic buffer at acidic pH (3.65), stained with Rose Bengal, is used as the basis of a simple and rapid agglutination test carried out on a tile and is very effective for identifying brucellosis in individuals within a group.

Materials and reagents

- White tiles (ceramic or plastic).
- Glass or wooden rods (tooth-picks).
- Shaker with rocking motion (approximately 30 rocks per minute).
- Antigen stained with Rose Bengal, available from commercial suppliers (Rhône-Mérieux, Commonwealth Serum Laboratories, Central Veterinary Laboratory) or prepared and standardised in the laboratory according to the method described by Alton et al [1]. It can be stored at 4°C but not frozen.
- Standard positive and negative sera.

Procedure

1. Bring the sera (test and standards) and the quantity of antigen necessary for tests being undertaken that day up to room temperature.
2. Place a spot (25 or 30µl) of each sera for testing on a white tile.
3. Mix the antigen thoroughly then place a spot of antigen, of equal volume, alongside each spot of serum (Figure 4.9, page 92).
4. Carefully mix each serum-antigen pair, using a clean rod for each sample, until a circular area of approximately 2cm diameter is obtained (Figure 4.10, page 92).
5. Rock the tile gently for 4 minutes, either manually or using a shaker with a rocking motion.

Reading and interpretation of results

Any agglutination visible to the naked eye after 4 minutes is considered as positive. Agglutination appearing after this delay is not considered positive.

Note

• The test is less sensitive if the serum and antigen are mixed immediately after removal from the refrigerator. The stock antigen, on the other hand, must be stored at 4°C to prevent deterioration.

4.6.2 Complement fixation

The complement fixation test (CFT) is unanimously acknowledged to be the most specific and sensitive technique for individual diagnosis of brucellosis in sheep and goats.

Principle

Complement is a complex protein that is able to attach itself to antigen-antibody complexes and induce cellular lysis in an standardised system. In the first stage of the CFT, the *Brucella* antigen and the test serum are mixed with fresh guinea-pig (complement) serum. If the test serum contains antibodies against *Brucella*, the complement becomes attached. In the second stage, sensitised red blood cells from sheep (i.e. mixed with antibodies raised against sheep erythrocytes or haemolysin) are added. If all the complement has been fixed during the first stage, no haemolysis occurs which signifies that the test serum contains antibodies. The reaction is positive. The presence of haemolysis indicates that the complement has not attached during the first stage because the serum does not contain antibodies against *Brucella*. The reaction is negative.

Materials and reagents

- 96 well microtitre plates (round bottomed wells).
- 12 head mechanical diluter to dispense 25µl volumes or multichannel pipette such as "Titertek" (Flow Laboratories) with disposable plastic tips for single use.
- Plate shaker.
- Centrifuge suitable for plates.
- Mirror for reading results.
- Antigen.
- Complement.
- Haemolytic serum.
- Sheep red blood cells.
- Veronal-Calcium-Magnesium buffer.
- Standard negative and positive sera of known titre.

Procedure

The four reagents used to measure the antibody titre of the test sera (the antigen, red blood cells, haemolytic serum or haemolysin and the complement) should be standardised before the test is carried out. Dilute antigen with veronal buffer according to the manufacturers instructions or following titration by the method of Alton et al [1]. The haemolytic complex is usually prepared from a 3% suspension of fresh sheep red blood cells mixed with an equal volume of rabbit serum raised against sheep red blood cells, at 2 to 5 times the minimum concentration required to produce 100% haemolysis in the presence of the complement. Titrate the complement separately in the presence [7] or in the absence [1] of antigen to determine the quantity of complement necessary to produce 50% lysis of a suspension of sensitised red blood cells. This quantity is known as the 50% haemolytic unit (HU_{50}) and 5 HU_{50} are used for the test.

The test proper, presented below, is undertaken in 96 well microtitre plates (round bottomed).

- Stage One

1. Place 50µl of each test serum in the first well (A) of each of the 12 rows of the required number of plates (Figure 4. 11).
2. Similarly, place 50µl of each of

the two standards, positive and negative, in the first wells (A) of rows 1 and 2 on the standards plate (Figure 4.11).

3. Cover the wells with an adhesive strip and inactivate the sera in a water bath at 62°C for 30 minutes.

4. Remove the adhesive strip and add 25µl of veronal buffer to each well except those in the first column (A) which already contain the serum.

5. Dilute the serum by repeated doubling dilutions using a 12 head mechanical diluter or a multichannel pipette (25µl) until the last wells (H) of the plate when the dilution will be 1:128.

6. Add 25µl of veronal buffer to the second well (B) of each row (anti-complement control for each serum).

7. Add 25µl of antigen, freshly diluted with veronal buffer (according to the manufacturers instructions or the result of titration by the method of Alton et al [1]), from the third (C) to the last (H) wells of each row.

8. Add 25µl of complement containing five 50% haemolytic units (HU_{50}) in each well except the first wells (A) in each row.

9. Complete the standards plate (antigen, complement and haemolytic complex controls) as follows (Figure 4.11):

• Antigen Control (wells A and B of row 3)

- 25µl of veronal buffer
- 25µl of antigen
- 25µl of complement containing 5 HU50

• Complement Control (wells C, D, E and F of row 3)

- 50µl of veronal buffer
- 25µl of complement containing (HU_{50})

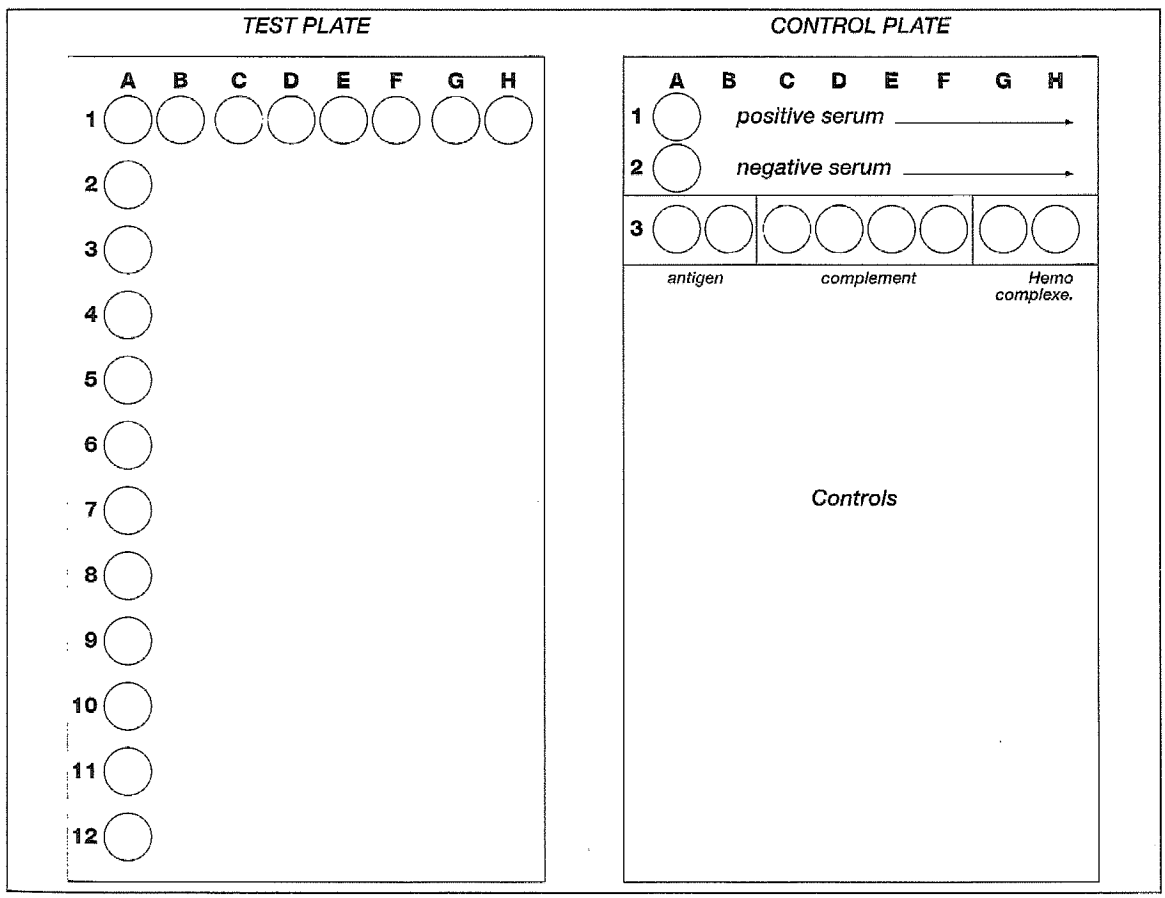

Figure 4.11 : Complement fixation test : Lay-out for a test plate and a control plate

• Haemolytic Complex Control (wells G and H of row 3)

- 75µl of veronal buffer

10. Gently tap each plate to mix the reagents, re-cover with an adhesive strip and place in an incubator at 37°C for 30 minutes (fixation in the warm) or at 4°C for 14 to 18 hours (fixation in the cold).

- Stage Two

11. Prepare the haemolytic complex by mixing equal volumes of a 3% red blood cell suspension and a haemolytic serum diluted in veronal buffer (according to manufacturers instructions or by the method of Alton et al [1]). Leave the mixture shaking at room temperature.

12. On removal of the plates from the incubator or refrigerator, add 25µl of haemolytic complex to wells B to H on the test plates and into all wells on the standards plate except for wells A in rows 1 and 2.

13. Re-cover the plates with an adhesive strip and incubate at 37°C for 30 minutes on a shaker (to prevent precipitation of red blood cells at the bottom of the wells).

14. On removal from the incubator, centrifuge the plates at 2000 rpm for 10 minutes at 4°C or, if a suitable centrifuge is not available, place the plates in a refrigerator for up to 2 hours to allow precipitation of all non-lysed erythrocytes.

Reading and interpretation of results (Figure 4.12, page 92)

Read the results from underneath the plates with the aid of a magnifying mirror or, if a mirror is not available, from above with the plate placed on a white light box. By either method the different controls, under satisfactory conditions, will show the following: Complete haemolysis (100%) in the antigen control and the complement control wells and absence of haemolysis in the haemolytic complex control wells. The results for each test serum are then scored from 0 (negative reaction) to 4+. With complete fixation (4+), relating to an absence of lysis, a button of red blood cells will be visible in the bottom of the well with a clear, colourless supernatant liquid. In the case of a negative reaction (score of 0) with complete lysis, no button of red blood cells will be visible and the liquid in each well will be clear and coloured by haemoglobin. The intermediate degrees of incomplete haemolysis are scored 1+, 2+ and 3+. The B well (anti-complement control) must show a complete haemolysis otherwise the result for the corresponding serum is not valid.

The serum titre is taken as the highest dilution showing a reaction of 1+ (or more), that is 25% (or more) fixation. A titre of 1:4 (or more) is regarded as positive or borderline. A titre of 1:8 is always considered as positive. The titre of a serum can also be expressed in International Units of Complement Fixation (IUCF) by comparison with anti-abortus international standard serum (available from CVL, Weybridge, UK) or a national equivalent. This standard serum, which is assigned an arbitary value of 1000 IUCF/ml, is used to establish the conversion factor which allows expression of a IUCF titre for an unknown serum using a given technique. If the titre (50% fixation or inhibition of haemolysis) of the standard serum is equal to 200, the IUCF/ml value of the unknown serum is given by the formula : 1000/200 x titre of unknown serum. The EC consider all sera with a titre equivalent to 20 IUCF as positive.

Note

• The reagents for complement fixation; antigens, complement, haemolytic serum, sheep red blood cells, veronal-calcium-magnesium buffer are available from commercial suppliers (BioMerieux) or can be prepared and standardised in the laboratory according to the protocols described by Alton et al [1].

• Complement fixation is a complex test which requires very careful preparation and standardisation of reagents. In particular, the complement, which is highly labile, must be titrated for each series of tests, preferably in tubes rather than on plates for the best determination of (HU_{50}) units. Each new batch of haemolytic serum must be systematically titrated.

• Anti-complementary reactions are not uncommon for sheep. Inactivation of the sera at 62°C and/or cold fixation allow a reduction of this phenomenon although this type of fixation increases the frequency and intensity of the prozone phenomenon. It is necessary therefore to take into account these characteristics when choosing the temperature of fixation.

4.6.3 Allergen test

The allergen test, together with serological diagnosis, increases the chances of detecting infection especially in flocks where the extent of *Brucella* infection is not clear. This test never gives false positive results and a positive result in a single animal always signals the presence of *Brucella* infection in the flock. On the other hand, all infected animals do not necessarily give positive results, making the test more applicable for identifying the stage of brucellosis infection in the flock than as a diagnosis of infection in individual sheep and goats. An important practical advantage of this test is that the results must be read and interpreted directly by the tester, which favours their co-operation in implementing prophylactic measures for control of brucellosis.

Principle

The allergen test is based on a delayed hypersensitivity reaction in animals inoculated with cytoplasmic proteins of rough and smooth *Brucella*. The initial allergens were prepared from filtrates of old boiled cultures and had the drawback of inducing antibody production and sensitisation of the animal. They have now been replaced with an allergen, devoid of LPS-S, the brucelline-INRA, prepared from part of one rough strain of *B.melitensis*, which is available commercially (Brucellergene OCB, Rhône-Mérieux).

Materials and reagents

- Syringe and needle for intramuscular injection.
- Callipers.
- Brucellergene OCB (Rhône-Mérieux).

Procedure

In sheep and goats the palpebral route is generally used for convenience of injection of the allergen and for assistance in the reading of the results. The recommended volume of Brucellergene OCB is 0.1ml, injected intradermally into the lower eyelid.

Reading and interpretation of results

The reaction is assessed 48 hours after injection, by comparison with the other eye. Any visible or palpable reaction on the test eyelid is considered positive. Intensity is assessed by the degree of oedema of the eyelid which generally causes a large swelling under the eye and is usually accompanied by lacrimation (Figure 4.13, page 93).

Note

- To avoid production of antibodies or sensitisation of the animal, it is essential that the allergen is of highest quality and contains no LPS-S.
- The allergen test cannot be used in flocks immunised with the Rev 1 vaccine as this induces a long lasting (2 years or more) hypersensitivity.

4.7 FUTURE WORK

The current techniques of direct (bacteriological) and indirect (immunological) diagnosis of brucellosis generally give satisfactory results when they are undertaken with rigorous control of the environment, reagents and procedures. However, the emergence of new biotypes of *Brucella*, particularly in marine mammals highlights the limitations of the present methods of identification and typing of the strains which do not always allow clear distinction between the different types of *Brucella* responsible for brucellosis in animals and man. The development of a simple system of molecular typing of *Brucella* is therefore necessary for the direct diagnosis of brucellosis and techniques studying the polymorphism of the genome (RFLP, PCR-RFLP) look promising for the future. In the area of indirect diagnosis, the strong association between serological (RB and CFT) and allergen tests usually allows efficient identification of infected flocks. Immunoenzymatic (ELISA) tests have been developed for many years and are used successfully on a wide scale in certain countries for the diagnosis of bovine brucellosis. Their application to diagnosis of ovine brucellosis would allow improved sensitivity and specificity of identification in comparison to those from RB and CFT but further work is still required in this area particularly in relation to the standardisation of reagents.

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