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Bovine Brucellosis

Bacteriology

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Contents

Bovine Brucellosis

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1. Introduction

In the past cultural examination for the diagnosis of bovine brucellosis was considered unreliable. The bacteria (*Brucella abortus*) are often present in the tissues only in small numbers and it has been difficult to obtain uncontaminated specimens. The development of highly selective media and the means for easy maceration of tissue have improved the prospects for successful cultures.

Bacteriological diagnosis can be used to determine the infection status of animals in problem herds and to validate serological procedures. It is axiomatic that isolation of *Brucella abortus* is the only criterion of a definitive diagnosis.

2. Culture of Brucella

Solid media are preferred for the culture of brucella as they facilitate recognition and discourage dissociation but liquid media permit the culture of larger volumes than can be conveniently dealt with on solid media.

2.1. Basal Media

A range of commercial dehydrated brucella media is available (see 6.1.). The medium we prefer is Trypticase soy (TS)(BBL Division of Becton, Dickinson & Co., distributed in Australia by Becton Dickinson, see 6.1.) broth to which is added 1.5% agar for solid medium. Bovine serum (5%) is required for the isolation and growth of *B. abortus* biotype 2 and is, therefore, added routinely. We use bovine serum but equine serum may be used. The serum should be free from brucella antibodies, and is inactivated at 56°C for 30 min and passed through a 0.22 µm sterilising filter. The medium is autoclaved, and cooled to 56°C before the serum is added.

Serum dextrose (SD) agar plates are used when specimens likely to contain *B. abortus* strain 19 are being examined. For this, nutrient agar is prepared, autoclaved, cooled to 56°C and 5 mL of a filter-sterilised stock solution of serum containing 20% dextrose, added per 95 mL of media.

2.2. Selective Media

The selective media may be stored for up to four weeks at 4°C in sealed containers. Preparation of stock solutions of the antibiotics is given in 6.2.

2.2.1. Solid Medium

In the preparation of selective agar medium (Kuzdas and Morse, 1953) the following substances are added per litre of basal medium: 100 mg of cycloheximide; 25 000 units of bacitracin and 6000 units of polymyxin B sulfate. Plates should contain at least 25 mL of medium.

2.2.2. Liquid medium

The antibiotics for the selective broth medium are similar to those of Brodie and Sinton (1975) and are given below, in quantities per litre of medium.

Cycloheximide	100 mg
Bacitracin	25 000 units
Polymyxin B sulfate	6000 units
Nalidixic acid	5 mg
Vancomycin	20 mg
Amphotericin B	1 mg
D-cycloserine	100 mg

At the concentrations listed above there is no inhibition of any *B. abortus* biotypes.

2.2.3. Biphasic medium

A medium consisting of both a solid and a liquid phase in the same bottle, similar to that described by Castaneda (1947) for blood cultures, is recommended for culture of materials such as milk and macerated tissues.

Sterile plastic tissue culture flasks (50 mL) are used [Disposable Products, 16 Park Way, Technology Park, SA 5099. Tel. (08) 349 6555; (008) 803 308. Fax (008) 806 073]. After the TS agar medium has been sterilised, cooled and the seven antimicrobial agents listed above and serum added, 8 mL are dispensed into each flask. The flask is laid flat on a narrow side while the medium solidifies. Freshly prepared selective broth is dispensed to the flasks in 9 mL amounts. Detachment of the agar from the side of the flask will not occur if the flask is held at room temperature (20–25°C) between setting of the agar and addition of the broth. These operations should be done no more than 24 hours apart.

2.3. Testing of Culture Media

It is essential to test the ability of each batch of culture medium to support the growth of *B. abortus*. This is best done by inoculating a few plates of each batch with a known number of cells (about 100) of the fastidious, serum-requiring *B. abortus* biotype 2. If the medium will support the growth of biotype 2 it will support all the other biotypes. The biphasic medium is inoculated with a similar number of cells of the same organism. For this a stock of freeze-dried culture containing a convenient number of brucella organisms per ampoule may be prepared. If this is not possible, appropriate dilutions of a 24 hour brucella culture can be made in peptone-saline and, if stored at 4°C these will give reproducible results up to one week after preparation. Whenever possible, testing of a new batch of medium should be done in parallel with a batch of known quality.

2.4. Incubation

All cultures are incubated at 37°C in air containing 5–10% carbon dioxide except for the SD agar plates used to isolate strain 19, which are incubated in air. Plates are incubated with the agar uppermost and flasks with the agar slope vertical. Flasks are incubated with the caps loose. Plates are examined after four and eight days. The flasks containing biphasic medium are

examined every four to seven days for up to 28 days. After examination, the flasks are tilted so that the liquid phase runs over the solid phase, then righted and returned to the incubator.

2.5. Identification of Isolates

Brucella colonies after four to five days incubation are round with smooth margins and are 2–4 mm in diameter. Viewed through transparent medium the colonies are translucent and a pale honey colour. Viewed from above the colonies are pearly white and convex. Brucella are Gram-negative coccobacilli and are stained red by the modified Ziehl–Neelsen method.

Tentative identification of isolates may be made using the slide agglutination test. A quantity of growth from an agar slope or portion of a large colony is dispersed in a drop of saline. To this a drop of diluted antiserum to smooth brucella is added and the mixture examined for agglutination. A control test using normal serum should be run to detect non-specific agglutination.

Isolates considered to be brucella on the basis of colonial morphology and slide agglutination tests can be submitted for typing. Send specimens to the National Brucellosis Reference Centre, Australian Animal Health Laboratory, PO Box 24, Geelong, Vic. 3220. Tel. (052) 275 000; Fax (052) 275 555.

3. Collection of Specimens for Cultural Examination

Success in culturing specimens depends on careful preparation and handling during transport. Specimens are collected using aseptic techniques and placed in sterile leak-proof containers of appropriate size to limit the amount of contained air which occupies unnecessary space and impedes refrigeration. For these reasons sterile plastic bags such as Whirl-paks (available from Nasco Industries Inc., Fort Atkinson, Wis., USA, distributed in Australia by Leader Products Pty Ltd, Craigieburn, Vic. 3064) are ideal for this purpose. Specimens should be refrigerated immediately they are collected and frozen if they cannot be cultured within 24 hours. For transport, the specimens are packed in stout polystyrene foam containers. If the specimens are already frozen they should be transported packed on dry ice to ensure that they remain frozen.

When sealing the Whirl-pak plastic bags, first squeeze out most of the air, then roll up the bag tightly around the wire and bend the wires over to maintain the seal.

3.1. Milk

Since infection of the udder may be confined to one quarter, 10–20 mL of milk is obtained from each quarter separately. The whole udder is washed and dried, and the tip of each teat is disinfected with a swab of 70% ethanol (C_2H_5OH). The first one or two streams of milk are discarded and then a sample from each teat is milked directly into a sterile container. Sterile plastic or glass jars or Whirl-pak plastic bags are suitable containers for milk. It is essential to avoid contact between the stream of milk and the milker's hand, both to protect the milker and to prevent cross contamination of specimens.

Resting mammary fluid is also valuable for bacteriological examination. After disinfecting the udder, all the fluid expressed from each teat is collected as there may be only a very small volume of secretion present.

3.2. Vaginal Swabs

Brucella may be recovered from a vaginal swab taken in the six-week period following parturition or abortion. A guarded sterile swab, e.g. sterile guarded mare swab (available from Cenvet, Crows Nest, NSW 2065, Australia) is used and the swab should then be broken off in a sterile container if it is to be cultured immediately or into a vial of transport medium (see 6.3.) if there is to be a delay before the swab is cultured.

3.3. Animal Carcases

The tissues from which brucella can most often be isolated are those of the reticuloendothelial system, plus either the contents of the pregnant or post-parturient uterus, the udder and its secretions, or the male reproductive tract (see 6.4. and 6.5.).

The equipment required for collecting the specimens, besides the usual protective clothing, gloves, knives etc., are:

(a) A steriliser and two perforated inner trays;
(b) 12 pairs of surgical scissors (15 cm), 12 pairs of rat-toothed forceps (15 cm) and two No. 4 scalpel handles and large blades;
(c) sterile pasteur pipettes or a 20 mL syringe and 18 gauge needles;
(d) a means of heating the steriliser such as a portable gas barbeque or stove-type gas burner;
(e) a gas torch for flaming the tissue;
(f) wet or dry ice, or an ample supply of 'cold bricks'; and
(g) sterile specimen containers.

After the animal is slaughtered and prior to removal of the skin the udder is removed, so that the mammary lymph nodes are removed with it. When removing the head, cut through the trachea 5–8 cm caudal to the larynx. This will ensure that the mandibular (syn. submaxillary) and medial retropharyngeal lymph nodes are left intact on the head.

Specimens for bacteriological examination are collected aseptically. For each tissue a separate set of sterile forceps and scissors is used. The tissue overlying and surrounding the lymph nodes or tissue to be collected is flamed prior to

removal of the specimen. After removal the specimen is placed in a sterile container.

When collecting lymph nodes they should be dissected free from the surrounding fat but every endeavor must be made to avoid cutting the capsule of the lymph node. Samples from the right and left sides are kept separate. A suitable specimen from the spleen would be about 10 g.

The tissues for cultural examination are listed in 6.4. and 6.5. The tissues are also listed in order of frequency of infection in infected animals (6.6. and 6.7.) (Corner *et al.*, 1987).

3.4. Foetus

From an aborted foetus or one taken from the uterus of a slaughtered reactor, samples of spleen, lung (both left and right) and stomach contents, 10–20 mL, are collected (see 6.4.). During collection the same aseptic precautions as described above are used. A sample of stomach contents may be withdrawn with a pasteur pipette or sterile syringe.

3.5. Foetal Membranes

One or two cotyledons are collected from the foetal membranes of a pregnant uterus of a slaughtered reactor or from the aborted membranes. In abortion due to brucella infection, parts of the foetal membranes may contain large numbers of brucella organisms; this is also true of membranes from infected full-term births. Brucella may also be isolated in large numbers from healthy looking cotyledons. Some infected cotyledons lose their normal bright red appearance and become a dirty greyish–yellow colour. The membranes should be examined carefully and the least healthy-looking cotyledons, or portions of them, should be removed and transferred to the laboratory for culture.

4. Culture of Specimens

Brucella are often present in very small numbers in tissues and milk, thus the chances of isolating the organism are improved by increasing the number of plates inoculated per sample or, more conveniently, by using a selective biphasic medium in addition to one or more plates of solid medium. For each specimen we inoculate one plate of solid selective medium, and two flasks of biphasic medium. The plate medium is included as it may give an earlier positive result than the biphasic medium. Macerated tissue or fluid specimen is inoculated onto the plate of solid medium using a sterile swabstick. The two flasks of biphasic medium are inoculated with a pasteur pipette; the first inoculated with 1 mL of inoculum and the second with 2 mL.

Calculations made from the results of culturing 8600 specimens from 440 reactor cattle by the above method, indicated that there was a 95% chance that an infected specimen would be detected. In addition, subsets of specimens which maximise the detection rate for a given amount of effort and using the same culture method were established for cows (see 6.8.) and heifers (see 6.9.). The detection rate might be increased either by increasing the volume of inoculum for specimens, or by culturing extra tissues in addition to those in the recommended subset. Because of the high sensitivity of the method, it seems better to include additional tissues than to use larger inocula from those in the subset.

For samples from cattle vaccinated with *B. abortus* strain 19 a plate of selective SD agar should be inoculated and incubated in air. Strain 19 grows better on SD agar plates than on TS agar plates but grows as well as virulent biotypes in the biphasic medium. A combination of SD agar plates and TS agar plates allows the identification of mixed infections of Strain 19 and virulent *B. abortus*.

For the initial culture, only half the sample is used, the other half being frozen for later examination in case the initial cultures become overgrown by contaminants. Where the specimen submitted is tissue, intact tissue should be stored frozen as the viability of brucella in frozen macerated tissue suspensions is poor. The number of viable brucella in tissues stored frozen at -20°C remains constant for at least 18 months. If unprocessed tissues that have been frozen and thawed are refrozen, then thawed a second time, there is a small loss of viability, but if the process is repeated the viability of the bacteria falls sharply (L.A. Corner and G.G. Alton, unpublished data 1982).

Frozen specimens are thawed at room temperature or overnight at 4°C.

4.1. Milk

The milk samples from each quarter are cultured separately to avoid failure to detect infection by the effects of dilution. Centrifuge 10–20 mL for 20 min at 6000–7000 *g*. After centrifugation collect a portion of the cream layer on a sterile cottonwool swabstick. Discard the skim milk and remaining cream and retain the sediment. Inoculate an agar plate by smearing a mixture of the cream and the sediment over the whole surface of the plate with the swabstick. After inoculating the plates, break off the swab into a flask of biphasic medium. Alternatively 1 or 2 mL of whole milk may be added to a flask of biphasic medium.

Gravity cream may be cultured. Milk samples in sterile test tubes or sterile universal bottles are held at 4°C overnight and the cream is cultured as described above. This method gives similar results to that described above but there is a risk that contaminants may proliferate in the sample during storage.

4.2. Other Liquids

Foetal stomach contents and synovial fluid are spread directly on solid selective medium with a swabstick and pipetted into biphasic medium. Culture of blood may also be attempted (see 6.10.).

4.3. Tissue

Tissues with gross surface contamination should be flamed or dipped in ethanol before flaming. Using sterile instruments the superficial fat is trimmed from the specimen. Half the tissue specimen or up to 10 g of the tissue is sliced into small pieces. The pieces are placed in a sterile plastic bag and macerated in 2–10 volumes of sterile isotonic phosphate buffered saline (PBS) pH 6.3 (see 6.12.) or nutrient broth, in a Colworth stomacher (FSE Pty Ltd). This operation is best conducted in a Class 2 biohazard cabinet. Other methods for the preparation of tissue for culture are listed in 6.11.

4.4. Foetal Membranes

Aborted foetal cotyledons frequently show gross contamination with soil or bedding and should therefore be washed before culture. They are rinsed in at least three changes of sterile saline or PBS pH 6.3 before being macerated. To decrease the risk of contaminants overgrowing the plate, 10-and 100-fold dilutions of the macerated tissues are cultured. The dilutions are made in PBS pH 6.3. Alternatively by using a swabstick, a small amount of inoculum from one plate can be applied to the second plate. Similarly a third plate can be inoculated from the second.

4.5. Vaginal Swabs

If the swab is submitted in a sterile tube, the end of the tube from which the swab is to be withdrawn is flamed, the swab removed and rubbed over the surface of the medium. The swab may then be placed in a flask of biphasic medium.

If the swab is submitted in transport medium, a plate is inoculated with the swab. The swab is then placed in a flask of biphasic medium.

4.6. Isolation of Brucella by Laboratory Animal Inoculation

Laboratory animal inoculation for the primary isolation of *B. abortus* is not recommended. Culture methods outlined above are at least as sensitive as animal inoculation.

5. References

Brodie, J., and Sinton, G.P. (1975). Fluid and solid media for isolation of *Brucella abortus*. *Journal of Hygiene* **74**(3), 359–67.

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6. Appendixes

6.1. Appendix 1 — Commercially Available Brucella Media

6.1.1. Trypticase–soy

BBL Division of Becton, Dickinson & Co. Cockeysville, MD USA. Agent: Becton Dickinson, 80 Rushdale Rd, Knoxfield, Vic. 3180. Tel. (03) 764 2444; Fax (03) 764 2550.

6.1.2. Bacto–tryptone

N.B. 'Peptone 140' is an equivalent product according to the manufacturer.

Difco Laboratories. Detroit, Michigan, USA. Agents: Helena Laboratories (Aust.) Pty Ltd, Mt Waverley, Vic. 3150. Tel. (03) 543 7299, (008) 033 137; Fax (03) 543 7542. FSE Pty Ltd, 47–49 Overseas Drive, Noble Park, Vic. 3174. Tel. (03) 795 0077; Fax (03) 790 1900.

6.1.3. Tryptic Soy Agar and Broth/Brucella Agar and Broth

Gibco Diagnostics Laboratories. Madison, Wisconsin, USA. Agents: Helena Laboratories (Aust.) Pty Ltd (see 6.1.2.).

6.1.4. Tryptone soya/Brucella medium

Oxoid (Australia). Heidelberg West, Vic. 3081. Tel. (03) 458 1311, (008) 331 163; Fax (03) 458 4759.

We prepare nutrient agar from Bacto peptone (Difco) and beef extract (Lab Lemco Powder, Oxoid). Dehydrated nutrient agars often called blood agar base or Columbia agar, are available from numerous commercial sources.

6.2. Appendix 2 — Preparation of Antibiotic Stock Solutions

Convenient stock solutions of antibiotics may be prepared as follows.

6.2.1. Cycloheximide

Sigma-Aldrich. Unit 2, Anella Avenue, Castle Hill, NSW 2154. Tel. (02) 899 9977; Fax (008) 800 096. Cycloheximide powder (1 g) is dissolved in 100 mL of distilled water and passed through a 0.22 µm Millipore filter. Add 10 mL of stock solution per litre of melted and cooled medium. Alternatively, the powder can be dissolved in 5 mL of acetone (CH_3COCH_3) and then diluted in distilled water to make 100 mL. No sterilisation is required. The solution is relatively stable and may be stored in a domestic refrigerator for up to six months.

6.2.2. Bacitracin

Sigma-Aldrich (see 6.2.1.); other brands. This antibiotic is supplied in bottles of sterile powder, or as tablets each containing 2500 units/mL of bacitracin, 10 mL is added per litre of medium. If the entire amount is not immediately required it may be divided into aliquots and stored, frozen, for up to two weeks.

6.2.3. Polymyxin B sulfate

Wellcome Pharmaceuticals, UK. Agents: Murex Diagnostics, 53 Phillips Street, Cabarita, NSW 2137. Tel. (008) 819 289; Fax (02) 743 6759. Calbiochem-Novabiochem Pty Ltd, PO Box 140, Alexandria, NSW 2015. Tel. (02) 318 0322, (008) 023956; Fax (02) 319 2440.

Dissolve 500 000 units (the contents of 1 vial) in 50 mL of water and add 0.6 mL/L of medium. Surplus stock solution may be kept frozen for up to one month; it should not be refrozen after thawing.

6.2.4. Vancomycin

Sigma-Aldrich, see 6.2.1. Dissolve in sterile water to give 50 mg/mL and add 0.4 mL of stock solution per litre of medium. Surplus stock solutions should be discarded.

6.2.5. Nalidixic Acid

Sigma-Aldrich, see 6.2.1. Dissolve in 0.5 mol/L sodium hydroxide solution at the rate of 5 mL/mL, then add 1 mL stock solution per litre of medium.

6.2.6. Amphotericin B

Fungizone, Squibb; Bristol-Myer-Squibb, Noble Park, Vic. 3174. Tel. (03) 213 4231; Fax (03) 701 1334.

Dissolve in sterile water at 1 mg/mL and add 1 mL of stock solution per litre of medium. Surplus stock solution can be stored in the dark at 4°C for up to six months.

6.2.7. Cycloserine

Sigma-Aldrich (see 6.2.1.); other brands. Dissolve in sterile water at 50 mg/mL and add 2 mL of stock solution per litre of medium. Surplus stock solutions should be discarded.

6.2.8. Commercial Antibiotic Mixture

A freeze-dried antibiotic supplement for the isolation of brucella species is available commercially (Oxoid Brucella Supplement SR83).

6.3. Appendix 3 — Brucella Transport Medium (D. Pietz, pers. comm. 1982)

Trypticase soy or Tryptone broth (0.5 mL) in a 100 x 13 mm capped tube.

After collection the tip of the swab is inserted into the tube and the shank of the swab cut off and the cap replaced. The tube is transported to the laboratory under refrigeration and a plate of medium inoculated with the swab which is then placed in a flask of biphasic selective medium.

6.4. Appendix 4 — Specimens Collected from Cows and Heifers

6.4.1. Head

(a) Parotid lymph node (l.n.) (left and right).
(b) Mandibular (Submaxillary) l.n. (left and right).
(c) Medial retropharyngeal l.n. (left and right).

6.4.2. Body

(a) Caudal superficial cervical (prescapular, suprascapular) l.n. (left and right).
(b) Subiliac (prefemoral, precrural) l.n. (left and right).
(c) Medial iliac (internal iliac) l.n. (left and right).
(d) Mammary (supramammary, superficial inguinal) l.n. (left and right).

6.4.3. Visceral Organs

(a) Caudal (posterior) mediastinal l.n.
(b) Mesenteric l.n. (duodenal, jejunal and ileal regions).
(c) Hepatic l.n.
(d) Spleen (5–10 g).
(e) Uterus (caruncles) or cotyledon.
(f) Udder (if lactating or has been lactating. Samples from all quarters.)
(g) Milk or resting mammary fluid (as for udder).

6.4.4. Foetal Tissues

(a) Spleen.
(b) Lung (left and right).
(c) Stomach contents (10–20 mL).

The following additional specimens may be collected for culture from particular animals: vaginal swab, blood and synovial fluid.

6.5. Appendix 5 — Specimens collected from Bulls (L.A. Corner, G.G. Alton and H. Iyer, unpublished data 1986)

6.5.1. Head

(a) Parotid l.n. (left and right).
(b) Mandibular l.n. (left and right).
(c) Medial retropharyngeal l.n. (left and right).

6.5.2. Body

(a) Caudal superficial cervical l.n. (left and right).
(b) Subiliac l.n. (left and right).
(c) Medial iliac l.n. (left and right).
(d) Scrotal (superficial inguinal) l.n. (left and right).

6.5.3. Visceral Organs

(a) Caudal mediastinal l.n.
(b) Mesenteric l.n. (duodenal, jejunal and ileal regions).
(c) Hepatic l.n.
(d) Spleen (5–10 g).
(f) Testes (left and right).
(g) Epididymis (left and right).
(h) Ampulla (left and right).
(i) Seminal vesicle (left and right)

6.5.4. Decreasing Order of Importance of Specimens from Bulls (Plant *et al.*, 1976)

(a) Ampulla.
(b) Mandibular l.n.
(c) Medial iliac l.n.
(d) Subiliac l.n.
(e) Caudal superficial cervical l.n.
(f) Medial retropharyngeal l.n.
(g) Seminal vesicle.
(h) Testis.
(i) Epididymis.
(j) Paratid l.n.
(k) Caudal mediastinal l.n.
(l) Spleen.

The following additional specimens may be collected for culture from particular animals: semen, blood and synovial fluid.

6.6. Appendix 6 — Specimens yielding Brucella abortus *from known infected cows (from Corner et al. 1987)*

See Table 1.

6.7. Appendix 7 — Specimens yielding Brucella abortus from known infected heifers (from Corner et al., 1987)

See Table 2.

6.8. Appendix 8 — Subset of tissues for cows that maximise detection of infection for a given amount of effort (from Corner et al., 1987)

See Table 3.

6.9. Appendix 9 — Subset of tissues for heifers that maximise detection of infection for given amount of effort (from Corner et al. *1987)*

See Table 4.

Table 1. Specimens yielding *Brucella abortus* from known infected cows (from Corner *et al.* 1987)

Specimen	*No. examined*	*No. positive on culture (%)*
Lymph nodes		
Parotid	123	60 (49)
Mandibular (Submaxillary)	122	61 (50)
Medial retropharyngeal	123	83 (67)
Caudal superficial cervical (prescapular)	123	65 (53)
Caudal mediastinal	122	37 (30)
Hepatic	87	15 (18)
Jejunal mesenteric	85	6 (7)
Medial iliac	123	89 (72)
Subiliac (prefemoral)	123	111 (90)
Mammary (Superficial inguinal)	123	111 (90)
Spleen	122	24 (20)
Udder	88	74 (84)
Uterine caruncle	101	47 (47)
Milk	102	86 (84)
Foetal tissue	19	5 (26)
Foetal membranes (cotyledons)	19	3 (16)

Table 2. Specimens yielding *Brucella abortus* from known infected heifers (from Corner *et al.*,1987)

Specimen	*No. Examined*	*No. Positive on Culture (%)*
Lymph nodes		
Parotid	61	39 (64)
Mandibular (submaxillary)	61	43 (71)
Medial retropharyngeal	61	38 (62)
Caudal superficial cervical (prescapular)	61	38 (62)
Caudal mediastinal	61	30 (49)
Jejunal mesenteric	61	8 (33)
Medial iliac	61	35 (57)
Subiliac (prefemoral)	61	38 (62)
Mammary (superficial inguinal)	61	34 (56)
Spleen	61	30 (49)
Liver	23	4 (17)
Lung	22	5 (23)
Kidney	22	5 (23)
Uterine caruncle	44	9 (21)
Udder	22	5 (23)
Milk	6	6 (100)
Foetal membranes	13	2 (15)
Foetal tissue	13	2 (15)

N.B. A heifer is defined here as an animal <18 months of age i.e. having no permanent incision teeth.

Table 3. Subset of tissues for cows that maximise detection of infection for a given amount of effort (from Corner *et al.*, 1987)

Tissue	*Cumulative No. detected (total 137)*
Mammary l.n[A]	121
Mandibular l.n.	129
Superficial cervical l.n.	132
Medial iliac l.n.	134
Parotid l.n.	135
Subiliac l.n.	136
Uterine caruncle or foetus or cotyledon	137

[A] l.n. = lymph node.

Table 4. Subset of tissues for heifers that maximise detection of infection for given amount of effort (from Corner *et al.* 1987)

Tissue	*Cumulative No. detected (total 137)*
Mandibular l.n.[A]	45
Parotid l.n.	49
Medial retropharyngeal l.n.	52
Uterine caruncle or foetus or cotyledon	55
Caudal superficial cervical l.n.	57
Mesenteric l.n.	58
Spleen	59
Medial iliac l.n.	60
Subiliac l.n.	61

[A] l.n. = lymph node.

6.10. Appendix 10 — Blood Culture

Blood may be cultured on solid medium, with or without preliminary treatment by freezing and thawing. However, the culture of blood is best done in liquid medium. The blood sample, about 10 mL, contained in the syringe or vacuum collecting tube is transferred aseptically to a biphasic medium flask. If blood is not mixed

with anticoagulant when drawn it must be transferred immediately to the biphasic medium flask and gently mixed with the broth containing 2% sodium citrate $C_6H_5Na_3O_7$).

After inoculation, the flasks are incubated in an upright position and examined every four to seven days for at least 28 days.

6.11. *Appendix 11 — Alternative Methods of Preparing Tissue for Culture*

If a Colworth stomacher [A.J. Seward, distributed by Townsend and Mercer Pty Ltd (branches in all States)] is unavailable the following procedures may be adopted. These are listed in decreasing order of merit (for cultural effectiveness).

(a) A tissue grinder (Griffith tissue grinder).
(b) A tissue blender (rotating blade type).
(c) Pestle and mortar, with or without the addition of sterile sand.
(d) Flame the sample; cut through the sample and rub the exposed surface on the agar plate.

6.12. *Appendix 12 — Phosphate Buffered Saline pH 6.3*

Phosphate buffered saline of pH 6.3 is prepared as follows. Dissolve 0.5 g sodium dihydrogen phosphate dihydrate ($NaH_2PO_4.2H_2O$), 0.22 g of anhydrous disodium hydrogen phosphate (Na_2HPO_4) and 8.5 g of sodium chloride in 1 L of distilled water.