

## Paratuberculosis (Johne's Disease)

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## SUMMARY

Paratuberculosis (Johne's disease) is a chronic enteritis of ruminants caused by infection with *Mycobacterium avium* subsp *paratuberculosis*.

### Identification of the agent

The diagnosis of Johne's disease is divided into two parts: the diagnosis of clinical disease, and the detection of subclinical infection, which is essential for control of the disease at farm, national or international level.

Diagnosis of Johne's disease is made on clinical grounds confirmed by the demonstration of *M paratuberculosis* in the faeces by microscopy, culture, or by the use of DNA-based techniques. Diagnosis is made at necropsy by the finding of the pathognomonic lesions of the disease in the intestines, either grossly or histologically and by isolation of *M paratuberculosis* using culture techniques.

The detection of subclinical infection depends on the detection of specific antibodies by serology, or culture of *M paratuberculosis* from faeces. The choice of test depends on the circumstances and the degree of sensitivity required at individual animal or herd level.

Cultures of *M paratuberculosis* may be obtained from faeces or tissues, after treatment to eliminate contaminants, by inoculation of solid or liquid media containing egg yolk and the specific growth factor – mycobactin – that is essential for the growth of *M paratuberculosis*.

### Serological tests

The single largest problem in Johne's disease control is the difficulty of detecting subclinically infected animals. The serological tests commonly used for Johne's disease are complement fixation, absorbed enzyme-linked immunosorbent assay and agarose gel immunodiffusion. Sensitivity and specificity are often determined by reference to results of faecal culture.

### Status of Australia and New Zealand

In Australia, bovine and ovine Johne's disease are restricted to parts of temperate south-eastern Australia in dairy and beef cattle, sheep, goats, alpaca and deer. Johne's disease is widespread in sheep, cattle and dairy goats in New Zealand. There is an emerging problem of Johne's disease in farmed deer in New Zealand.

## Introduction

Johne's disease (JD) is caused by *Mycobacterium avium* subsp *paratuberculosis* (*M paratuberculosis*). It is a chronic enteritis of ruminants, which is usually characterised by a lengthy incubation before producing clinical disease. Several excellent reviews of the topic have been published.<sup>1-4</sup> Johne's disease is a notifiable disease in all States and the Northern Territory in Australia. Johne's disease is no longer a reportable disease in New Zealand.

### Aetiology

*Mycobacterium paratuberculosis* is an aerobic, non-spore forming, Gram-positive, non-motile, acid-fast bacillus that is a slow growing intracellular parasite. It is closely related to *M avium* and the wood pigeon bacillus *M silvaticum*. Only a 1.2% base substitution separates *M avium* and *M paratuberculosis* with the distinguishing feature between these organisms being the presence of 15–20 copies of the insertion sequence IS900 in the *M paratuberculosis* genome. *M paratuberculosis* can be separated from both *M avium* and the wood pigeon bacillus by DNA techniques such as restriction endonuclease analysis, restriction fragment length polymorphism (RFLP) analysis,<sup>5, 6</sup> pulsed field gel electrophoresis and field inversion gel electrophoresis. Separation of these organisms into 3 distinct clusters was confirmed in a large numerical taxonomy study based on phenotypic tests.<sup>7</sup> Thorel *et al*<sup>7</sup> subsequently proposed new names for these three clusters; *M avium* subsp *avium*, *M avium* subsp *paratuberculosis* and *M avium* subsp *silvaticum*. Nomenclature continues to be debated and most scientists agree on the continued use of *M paratuberculosis* or MAP as an abbreviation for this organism.

A study of *M paratuberculosis* isolates in New Zealand demonstrated distinct cattle and sheep RFLP patterns<sup>8</sup> and an intermediate pattern has been identified in organisms from sheep in Canada and South Africa.<sup>9</sup> Pigmented isolates have been reported in Britain and RFLP analysis has identified other minor variations. Some sheep strains, including those in Australia, have been notoriously difficult to culture on existing solid medium.<sup>10</sup> It is now known that sheep strains of *M paratuberculosis* can be grown in the liquid Middlebrook (BACTEC 12B) medium used for cattle strains provided additional egg yolk is used. A new solid medium based on the successful 12B formulation is now available for culture of sheep strains.<sup>11</sup>

### Epidemiology

Johne's disease affects livestock welfare and productivity by way of direct effects on growth and production and indirectly through restrictions on trade. It occurs in a range of animal species, especially ruminants.

Johne's disease is endemic in cattle and dairy goats in the south-eastern states of Australia. In recent years, reviews of the impact of Johne's disease on the Australian cattle industries have generally recognised that the disease is spreading and that, although direct economic effects in most infected herds may be relatively low, restrictions on livestock trade and potential market losses are significant, particularly in elite breeding herds.

Following the first diagnosis of Johne's disease in alpaca in 1993<sup>12</sup> extensive testing of these animals has detected further related cases.

Ovine Johne's disease was first diagnosed in the central tablelands of NSW in 1980 and a control programme instigated in the late 1990s has identified an increasing number of infected sheep flocks in this region. Tracing investigations also found infected sheep flocks in Victoria, Flinders Island and Kangaroo Island and since 2000, infected flocks have also been found in south-eastern South Australia and on two properties in mainland Tasmania

Johne's disease has also recently been diagnosed in deer in Victoria and South Australia.

In September 2001, there were approximately 1,368 known infected herds of cattle in Australia. The known herd prevalence among dairy herds in south-eastern Australia is about 15%. At the same time, there were 689 infected sheep flocks, 17 infected goat herds, 4 infected alpaca herds and 5 infected deer herds. Western Australia has conducted comprehensive testing to demonstrate freedom from Johne's disease in cattle, alpaca, sheep and goats, and is accepted as being a free area for Johne's disease. Endemic infection is not known to occur in Queensland or the Northern Territory.

The first case that was ever shown to be Johne's disease in New Zealand occurred in an imported cow in 1912. Johne's disease is now endemic in dairy cattle in New Zealand. Ovine Johne's disease was first reported in New Zealand in South Canterbury in 1952. Subsequently the disease has become endemic in sheep flocks throughout both islands. Johne's disease was first recognised in farmed deer in the late 1980s and currently over 200 infected herds have been identified.

Although natural infection of cattle and sheep is usually associated with cattle and sheep strains of *M*

*paratuberculosis* respectively, cross-infections have been reported.<sup>8</sup> Cattle ('C') strains have been isolated from cattle, goats, alpaca, deer, sheep and a black rhinoceros in Australia and from cattle, goats and farmed deer in New Zealand. Based on cultural characteristics, sheep in Australia and New Zealand are generally considered to be infected by a different type of *M paratuberculosis* to those commonly infecting cattle, goats and alpaca. Two extensive Australian studies failed to reveal any significant occurrence of sheep strain infecting cattle.<sup>13,14</sup> While retrospective studies using direct PCR have shown that sheep ('S') strains are capable of causing disease in cattle,<sup>15</sup> in Australia this probably requires close contact between infected sheep and calves<sup>15</sup>. Recent evidence suggests that kangaroos and wallabies can become infected with *M paratuberculosis* if grazing closely with heavily infected sheep. It is unknown whether these macropods play any role in transmission of infection back to sheep. (P. Cleland, personal communication)

The most important source of infection is faeces from animals with *M paratuberculosis* infection. Early in the disease shedding in faeces may be intermittent. The number of organisms in faeces increases as the disease progresses and may increase when infected animals are subjected to stress. Most animals become infected by ingesting the organism in contaminated feed or water. Cattle are usually infected as young calves and develop resistance to infection with age. Cattle older than 12 months are relatively refractory to experimental infection while sheep and goats remain susceptible and are not known to develop a natural resistance with age. The survival of *M paratuberculosis* in the environment is favoured by low temperatures, moisture and protection from solar radiation. Some animals may become infected *in utero* and the chance of this occurring increases as the disease progresses in the dam. While *M paratuberculosis* has been isolated from milk, the number of organisms shed in the milk is orders of magnitude less than that found in the faeces.

### Clinical Signs

Clinical Johne's disease in cattle typically presents as a syndrome of chronic and progressive emaciation and persistent diarrhoea. The faeces are usually green and bubbly and do not contain blood or mucus. Faecal consistency may improve for short periods and then diarrhoea may return with increased severity. Affected animals are bright and alert and eat well throughout the course of the disease but in advanced cases, submandibular oedema may be observed. On rectal examination the mucosa may feel thickened or corrugated. The age of onset of clinical signs can be quite variable. In most cases, clinical signs of Johne's disease do not appear until animals are more than 3 – 4 years of

age but in some herds the onset of disease has been seen in 2-year-old animals.

Differential diagnostic possibilities for Johne's disease in cattle include:

- chronic fascioliasis
- gastro-intestinal parasitism
- enzootic bovine leucosis
- mucosal disease
- copper deficiency
- left displacement of the abomasum
- lipomatosis (fat necrosis)
- tumours of the gastro-intestinal tract
- salmonellosis
- coccidiosis
- carbohydrate engorgement
- yersiniosis

The predominant clinical signs of Johne's disease in sheep, goats and alpaca are weight loss and emaciation. Diarrhoea is usually absent or limited to soft, pasty faeces. In sheep flocks the first indication of disease may be the development of a distinct 'tail' in the mob. This 'tail' is demonstrated as the weaker animals that drop toward the tail (back) of the mob when the animals are mustered.

A notable feature of Johne's disease in farmed deer is occurrence of outbreaks of clinical disease in animals as young as 12 months old. Affected animals may have a short illness with weight loss and diarrhoea. Sporadic cases of clinical disease are also observed in older deer.

### Pathology

Although animals with advanced Johne's disease may have bacteraemia, the only specific lesions are found in the intestine and associated lymph nodes. Early during the course of *M paratuberculosis* infection, gross lesions may not be evident but, in clinical cases, the mesenteric lymph nodes are enlarged, pale and oedematous. Some degree of lymphangitis is always present and the lymphatic vessels are often visible as 1 – 2 mm cords tracing from the intestines to the mesenteric lymph nodes. Often, lymphangitis is the only recognisable gross change. In sheep, goats and alpaca, the lymphatic vessels may be knotted and corded. Approximately 10% of clinically affected goats show caseation and calcification of the lymph nodes. In sheep and goats, some lesions may be visible as white foci 1 – 4 mm in diameter. In alpaca, enlargement of

all mesenteric lymph nodes is striking. A notable feature of Johne's disease in farmed deer is macroscopic pyogranulomas in mesenteric lymph nodes that are very difficult to distinguish from lesions caused by *M bovis* or other members of the *M avium* complex. In fact, many of the diagnoses of Johne's disease in deer in NZ are made by culturing the causative organism from samples sent as suspect tuberculosis cases.

In all host species, specific intestinal lesions are usually more developed in the lower jejunum and ileum. The ileocaecal valve may be enlarged, but the presence of specific lesions in the valve and immediately adjacent tissues is not constant and a wider range of specimens must be examined to ensure a reliable diagnosis. The classical intestinal lesion is diffuse thickening of the intestinal mucosa with development of transverse folds or corrugations. When these mucosal folds are well developed they cannot be smoothed out. The crests of the rugae may be congested and the mucosal surface is velvety. Necrosis rarely occurs in cattle and unlike sheep, goats and deer, there is no caseation or calcification.<sup>16</sup>

### Diagnostic Tests

Overviews of the advantages and disadvantages of a range of diagnostic tests, including immunodiagnosics, have been previously reported.<sup>17, 4</sup> Diagnostic tests are aids to diagnosis and results should be interpreted in the light of epidemiological, clinical and pathological findings. As the accuracy of diagnostic tests for Johne's disease is limited and influenced by host factors and the level of exposure to *M paratuberculosis* and other related bacteria in the environment, results should be interpreted in the context of the actual population under test. False negative and positive results will occur. The precision of any test is also limited so good quality control in specimen collection, handling, storage and laboratory testing is needed to maintain precision at a high level.

### Histopathology

The basic lesion is a cellular infiltration of the intestinal mucosa in the virtual absence of necrosis, hyperaemia or fibrosis. In all species, a wide spectrum of cellular responses has been described. In advanced cases with obvious gross lesions, the histological changes are obvious and very characteristic with acid-fast bacilli readily identifiable, but in animals with subclinical or limited infections, the changes are less distinct. In these cases, there may be a diffuse infiltration of the lamina propria with lymphocytes, plasma cells and eosinophils with occasional epithelioid cells often in the tips or the bases of the villi.

These changes may be more evident in the submucosa and in association with the lymphatic tissue. With progression of the disease, epithelioid cells increase in number and organisms are more readily detected. The cell accumulations are progressive and gradually compress and obliterate the crypts thereby contributing to the gross thickening of the intestine. Lymphatic tissue may have a diffuse distribution of the epithelioid cell clusters or may be in microscopic foci. Langhans' giant cells with typical peripheral nuclei are present following the fusion of large epithelioid cells.

Changes in the lymphatic tissue are a consistent finding. Early in the infection, lymphatic vessels are surrounded by lymphocytes and plasma cells and may contain clumps of epithelioid cells in the lumen. These changes are progressive, resulting in epithelioid granulomas that form in the wall and project into the lumen. Some necrosis is common in these nodules. Specific lesions in the lymph nodes are similar in character to those in the intestine. At first, the subcapsular sinus is lightly infiltrated with epithelioid cells and there is histiocytic metaplasia of medullary reticular cells. The infiltrations are progressive, forming focal or diffuse areas of epithelioid and giant cells in the paracortical areas.

Microscopic focal granulomas have been described in liver, tonsil, other lymph nodes and occasionally in kidney and lungs.

In animals with clinical disease, vast numbers of acid-fast bacilli may be observed both intracellularly and extracellularly in lesions using the Ziehl-Neelsen stain. These are called multibacillary cases. In preclinical cases however, acid-fast bacilli may be infrequent and difficult to detect<sup>1, 16, 18</sup> (paucibacillary). In sheep, some advanced cases have granulomatous lesions with very few acid-fast bacilli.

At necropsy, a standard set of tissues (Appendix 2) is collected into 10% buffered formalin, processed routinely and stained for normal tissue elements with haematoxylin and eosin and also for acid-fast bacilli using the Ziehl-Neelsen method.

A positive diagnosis of lesions consistent with *M paratuberculosis* infection is indicated if in any one section, one or more single giant cells and/or one or more accumulations of three epithelioid macrophages are observed in the intestinal lamina propria and/or lymph node cortex with the presence of at least one acid-fast bacillus (New Zealand requires the sighting of three acid fast bacilli) morphologically consistent with *M paratuberculosis*.

A finding of suggestive of *M paratuberculosis* infection is indicated if in any one section, two single Langhans' giant cells and/or two accumulations of three epithelioid macrophages in the intestinal lamina propria and/or lymph node cortex are observed without the detection of an acid-fast bacillus.

Tissues must be well preserved and sufficient time must be devoted to the search for acid-fast bacilli. From a single animal, each Ziehl-Neelsen stained section of tissue with cellular changes indicative of Johne's disease should be examined under oil immersion for a minimum of 10 min before reporting no evidence of acid fast organisms. If, after examination of sections from all tissues, it is only possible to make a diagnosis of suggestive of *M paratuberculosis* infection, examination of further sections from the blocks with lesions is recommended.

**Histopathology of Johne's disease in deer.** The histological changes in deer lymph nodes found in Johne's disease are often indistinguishable from those caused by *M avium* and often similar to those caused by *M bovis*. In both cases caseous necrosis with mineralisation is observed, changes which are also present in lesions caused by *M bovis*. Given that all three infections are not uncommon in New Zealand, histopathology can only give an indicative diagnosis of Johne's disease in deer.<sup>19</sup>

### Bacteriology

Examination of ZN stained faecal smears can be applied in clinical cases to demonstrate typical clumps of acid-fast bacilli. However, false negative results occur and while the presence of acid-fast bacilli in clumps of 3 or more cells morphologically consistent with *M. paratuberculosis* is consistent with Johne's disease, this is not a definitive test. The isolation of *M paratuberculosis* from faeces or tissue is the definitive test for diagnosis of Johne's disease. The faecal culture test relies upon the animal shedding sufficient numbers of bacteria to be detected. In sheep, faecal specimens from up to 50 individuals can be pooled and culture undertaken successfully on the pooled sample. This pooled faecal culture (PFC) approach is used as a screening test in sheep flocks and in the sheep Market Assurance Program. Pooled faecal culture has been examined in the Netherlands for cattle using smaller numbers of animals and may have application to other animal species.

Culture requires decontamination of the specimen, preparation of special medium containing mycobactin and incubation periods of 2 – 6 months. Hexadecylpyridinium chloride (HPC) is recommended as the decontaminant of

choice. The most common solid medium used for primary isolation of *M paratuberculosis* is Herrold's egg yolk agar containing mycobactin (HEYM: Appendix 1). The addition of sodium pyruvate has been reported to inhibit the growth of some strains, but for most strains the number and size of colonies recovered is increased. A variety of synthetic media are available for growth of adapted strains and the production of Johnin, antigens and vaccine (for example, mycobactin-containing Middlebrook or Watson-Reid media<sup>20</sup>). Until recently, only a small number of successful isolations from sheep have been made using Herrold's medium in Australia and New Zealand. Liquid medium improves the isolation of *M paratuberculosis* from sheep and a solid medium (modified Middlebrook 7H10: Appendix 1) is now also available.<sup>21, 11</sup>

There are two techniques for culturing samples for *M paratuberculosis*: i) conventional culture on solid media, and ii) radiometric culture (BACTEC™) where growth in liquid medium is identified by the detection of radiolabelled metabolites. The latter method reduces the time required for obtaining a result and is considered to be a more sensitive technique. Specimen preparation involving filtration or double incubation and centrifugation may improve culture sensitivity.

Decontaminated faeces or tissues inoculated onto HEYM containing pyruvate will produce visible colonies of the cattle strain of *M paratuberculosis* after 6 – 8 weeks if the animal is shedding a large number of organisms and as long as 20 weeks in faeces of light shedders. Visible colonies of the sheep strain of *M paratuberculosis* appear after 2 – 9 weeks on modified 7H10 medium if subcultured from the liquid medium, but may take up to 20 weeks to grow if primary inoculation is onto 7H10 medium.

Colonies of the cattle strain of *M paratuberculosis* on Herrold's medium are typically convex, off-white to cream or buff coloured and non-chromogenic. As cultures age and media dries out, colonies and media tend to become more buff or beige-coloured and more raised. Colonies are soft, moist, glistening, non-mucoid and remain miscible with water. Colony size is initially pinpoint, and many remain at 0.25 to 1 mm, and tend to remain small when colonies are numerous on a slope. Older isolated colonies may reach 2 mm. On modified 7H10 medium, colonies of the cattle strain are less convex than those on Herrold's, especially in aged cultures, are pinpoint to approx 1 mm in diameter, and being buff-coloured, are only slightly lighter than the media. Compared with colonies of cattle strains on Herrold's, those on

7H10 are more difficult to detect (due to less contrast in colour between colony and medium) and to differentiate from colonies of some other mycobacteria.

Colonies of the sheep strain of *M paratuberculosis* on modified 7H10 are convex, soft, moist, glistening, off-white to buff, and very similar to the colour of the media. Colonies are typically between pinpoint and 0.5 mm, but can reach 1 mm, and rarely 1.5 mm if few colonies occur on a slope.

Saprophytic mycobacteria may have a similar appearance on either medium but are often evident after 5 to 7 days. Other organisms may grow on both media with colonies appearing after days or months.

Confirmation testing of colonies suspected to be *M paratuberculosis* as acid-fast bacilli involves staining thin smears of growth by the Ziehl-Neelsen method (Appendix 3) and examination under oil immersion (X 1000).

Mycobactin dependency can be used to differentiate *M paratuberculosis* from other mycobacteria although some *M. avium* have also been reported to demonstrate a requirement for mycobactin.<sup>22, 23</sup> Since mycobactin is present in the cell wall of the organism, a heavy inoculum may contain enough mycobactin to support the growth of a mycobactin-dependent mycobacterium on non-supplemented media. It is therefore very important to use a light inoculum. Assessment of mycobactin dependency can also be influenced by media pH and iron concentration.<sup>24</sup>

#### DNA Detection

*M paratuberculosis* may be identified from colony growth or from liquid radiometric culture medium by a polymerase chain reaction (PCR) targeting the insertion sequence IS900 of *M paratuberculosis* followed by restriction enzyme analysis (REA) of PCR product.<sup>25, 26</sup> *Mycobacterium* genus-specific primers can be added to the PCR to produce a multiplex PCR which provides a useful internal control during direct detection procedures for *M paratuberculosis* in BACTEC cultures.<sup>27</sup> Rapid typing of isolates as cattle ('C') or sheep ('S') strain may be undertaken by amplification and restriction of the IS1311 gene.<sup>28, 29</sup>

Methods for the direct detection of *M paratuberculosis* in faecal and tissue specimens using PCR have been evaluated as alternatives to culture.<sup>30, 31</sup> This test works well but is considered less sensitive than culture and is generally recommended when there are visible acid-fast bacilli. Direct detection of *M*

*paratuberculosis* DNA has been reported in formalin-fixed tissue sections and this method can also be used for strain typing.<sup>28</sup>

#### Intradermal Test

Intradermal testing for Johne's disease is no longer widely used and is not recommended for diagnosis due to its poor sensitivity and specificity in individual animals.<sup>32</sup> The test is still required by some overseas countries for certification of cattle or sheep. It is conducted in the same manner as the tuberculin test using purified protein derivative (PPD) reagents, Johnin PPD or *M avium* PPD (25,000 units/mL. CSL Limited, Parkville Victoria). Johnin PPD is not currently available from CSL and is unlikely to be available in the future. If Johnin is mandatorily required by the importing country, then it should be sourced from that country (or the country provides a source) subject to approval from Australia's Regulatory Authorities. The test is conducted by intradermal inoculation of 0.1 mL PPD into the caudal fold or the mid neck region. The test is read after 72 h; an increase in skin thickness of >3 mm being taken as positive.

#### Interferon- $\gamma$ Assay

A kit-based bovine interferon- $\gamma$  (IFN- $\gamma$ ) assay, developed to detect cell-mediated immunity to *M bovis*, is available from CSL Limited (BOVIGAM<sup>TM</sup>). The assay uses a monoclonal antibody and can detect picogram quantities of IFN- $\gamma$  in bovine blood after in vitro stimulation. It is marketed in Australia for bovine tuberculosis detection, and includes *M bovis* PPD and *M avium* PPD. This test is undergoing further evaluation and refinement for use in the diagnosis of Johne's disease. Some preliminary evaluations have been reported.<sup>33-37</sup>

Other tests have been used to assess CMI responses but have never been widely accepted as diagnostic tools. These test include: intravenous johnin;<sup>38</sup> lymphocyte transformation/stimulation/proliferation tests;<sup>18, 39-41</sup> and the leucocyte migration inhibition test.<sup>42-44</sup>

#### Agar Gel Immunodiffusion Test (AGID)

The AGID on serum has a high specificity, is relatively independent of the animal species and is simple and relatively inexpensive to perform. However, it uses large amounts of reagent, requires subjective interpretation and appears to have a low sensitivity (10–30%), particularly in cattle and goats. The test is widely used in sheep and goats but not in cattle. In sheep, the AGID is highly specific (98-100%) and reports of the sensitivity in ill-thrifty sheep vary from 78 to 93%.<sup>45, 46</sup> The AGID has been proven to have a very high positive predictive value for

histopathological evidence of paratuberculosis in goats.<sup>47</sup>

### Complement Fixation Test (CFT)

The use of the CFT in Australia as a standard serological test for Johne's disease in cattle is declining, due to its limited sensitivity and specificity. It is no longer recommended for diagnosis or certification. The test is performed using *M avium* strain D4,<sup>48</sup> which is more readily cultivated and gives similar reactivity to antigens prepared from *M paratuberculosis*. Despite its shortcomings, many countries still require a negative CFT before granting permission to import cattle. The recommended CFT method (Appendix 8) follows the well-standardised procedure published in the standard procedure for Bovine Brucellosis.

### Absorbed ELISA (Ab-ELISA)

The ELISA is at present the most sensitive and specific test for serum antibodies to *M paratuberculosis*. Its sensitivity is comparable with the CFT in clinical cases, but is greater in subclinically infected cattle and goats. The specificity of the ELISA is increased by pre-absorption of sera with *M phlei*.

### Ab-ELISA for Cattle

Three Ab-ELISA are currently approved for use in cattle in Australia, and one test is in use in NZ. Two of the approved tests were developed in Australia and one in France. A summary of the major components of the Ab-ELISA used is provided in Appendix 9. In each of the tests, an absorption step using *M phlei* is performed on diluted sera prior to testing in an indirect ELISA.<sup>49</sup>

The sensitivity and specificity of the Ab-ELISA is superior to the CFT.<sup>50, 51</sup> The Ab-ELISA has a high specificity, having been evaluated at 96.2-99.8% using sera from uninfected cattle.<sup>52, 51, 53, 54</sup> However, sensitivity and specificity estimates are not fixed and are dependent on the type of populations under test.

The sensitivity of the Ab-ELISA in adult subclinically infected cattle has been estimated at about 50%.<sup>50</sup> However, sensitivity in low shedder cattle can be as low as 15%,<sup>55, 56</sup> and a recent review of the literature on the sensitivity of the Ab-ELISA in cattle has estimated a sensitivity of 30% for low prevalence herds (A. Cameron, personal communication). Cut-off points for these assays are based on comparison of the optical density of the test sera with the negative or positive control by difference or ratio. The cut-off point is usually set to give a high specificity.<sup>57</sup>

### Ab-ELISA for Other Species

Assays based on the EMAI ELISA and the CSL EIA (PARACHEK™) for cattle have been evaluated in goats<sup>58</sup> and the PARACHEK™, EMAI and NZ ELISA have been evaluated in sheep.<sup>59, 45</sup> These ELISA are now approved tests for use in sheep and goats. Tests for alpaca and deer are under development and evaluation.

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This edition incorporates the advances in technology and standardisation since 1993 and accommodates the situation and practices in New Zealand.

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## APPENDICES

### Appendix 1

#### Media and Reagents

##### Mycobactin J

Mycobactin J stock solution (500 µg/mL) and working solution (50 µg /mL)

Mycobactin J 2 mg Allied Monitor, 201 Golden Drive, Fayette, Missouri, USA , 65248)

Add to the bottle supplied:

Ethanol 95% v/v 1 mL

Mix until completely dissolved.

Add 3 mL MilliQ purified water for stock solution or 39 mL water for working solution. Autoclave and store in the dark.

##### Malachite green

Prepare a 2% solution, autoclave, and add to medium through a 0.22 µm filter.

##### Egg yolks

Use fresh eggs from chickens that are not receiving antibiotics. If the eggs are dirty, scrub with detergent. Rinse with water and allow to dry. Soak in 70% ethanol for 30 min. Remove eggs and allow to dry in a sterile environment. Using aseptic technique, crack the egg and separate the egg white and chalaza from the yolk leaving the yolk in the shell halves. Rupture egg yolk before addition to media. Dispense aseptically into sterile, sealable bottles. To test sterility, a sample from each bottle should be cultured on blood agar and examined after incubation at 37°C for 48 h.

##### Herrold's Egg Yolk medium (with mycobactin J and sodium pyruvate) (HEYM)

This medium is for primary isolation of cattle strains of *M paratuberculosis*.<sup>60, 61</sup>

Proteose peptone	9.0 g
NaCl	4.5 g
Agar	15.0 g
Beef extract	2.7 g
Glycerol	27.0 mL
Sodium pyruvate	4.0 g

Dissolve the above in 890 mL distilled water, adjust pH of warm media to 6.9 to 7.0 with NaOH to achieve pH 7.2 in the solid media. Add 4 mL of stock solution (2 mg) of Mycobactin J. Autoclave at 121°C for 20 min. After cooling, aseptically add:

Egg yolks	120 mL
Malachite green (2%)	5.1 mL

Mix gently and dispense 10 mL volumes into sterile tubes to form slopes.

##### PANTA PLUS

Instructions from the manufacturer (Becton Dickinson) state that PANTA PLUS is reconstituted by adding 5 mL of reconstituting fluid (polyoxyethylene stearate aqueous solution; 4 mg/mL) to lyophilised PANTA supplement. In some laboratories, 10 mL of reconstituting fluid is used, and therefore double the standard PANTA PLUS volume is added to the final media.

## HPC/BHI

This solution contains 0.9% hexadecylpyridinium chloride (HPC) in half-strength Brain Heart Infusion (BHI).

Hexadecylpyridinium chloride	9 g (Sigma)
Brain heart infusion	18.5 g (Difco)
MilliQ purified water	1 L

Mix and dissolve (heating at 58°C may be required) then autoclave at 120°C for 20 min. Solution will froth so fill flasks to only 70% capacity. This solution is stable at room temperature for 1 week only. Do not refrigerate.

## VAN

(Vancomycin\* 100 µg/mL, Amphotericin B 50 µg/mL and Naladixic acid 100 µg/mL).

\* It is important to avoid contact with Vancomycin and gloves should be worn at all times.

## VAN

MilliQ purified water	200 mL
Vancomycin	20 mg
Naladixic acid	20 mg
Amphotericin B solution (10 mg/mL)	1 mL

Mix ingredients then dispense and store at -20°C.

For VAN/BHI add antibiotics to sterile half-strength Brain Heart Infusion instead of water.

## Amphotericin B stock solution (10 mg/mL)

Amphotericin B 45%, Sodium deoxycholate 35% (Sigma) 50 mg

Add 5 mL MilliQ purified water, mix to dissolve, dispense into aliquots and store at -20°C.

## BACTEC 12B vials

These are supplemented for growth of sheep and cattle strains of *M paratuberculosis*.

BACTEC 12B vials are stored at 4°C. The following supplements are added to each vial to give final concentrations of 0.8 to 1 µg/mL of Mycobactin J and a minimum of 16-17% egg yolk in a final volume of 5 to 6 mL.

	6 mL volume	5 mL volume
Mycobactin J (50 µg/mL <i>working solution</i> )	0.1 mL	0.1 mL
PANTA PLUS*	0.1 mL	0.1 mL
Egg yolk	1 mL	0.8 mL
Water	0.8 mL	nil

\*Where PANTA PLUS is reconstituted using 10 mL reconstituting fluid, 0.2 mL of stock solution is added and 0.1 mL less water.

Positive and negative controls should be set with each group of samples and read at weekly intervals.

## Modified 7H10 medium with Mycobactin J

This medium is for primary isolation of sheep or cattle strains of *M paratuberculosis*

Middlebrook 7H10 agar	19 gm
Casitone	1 gm
Glycerol	5 mL
MilliQ water	900 mL

Autoclave at 121°C for 15 min, cool to 58°C.

Using aseptic technique, combine the following additional ingredients adding the egg yolk last:

PANTA PLUS	50 mL
Mycobactin J solution (50 µg /mL)	25 mL
ADC enrichment	100 mL
Egg yolk	250 mL

Thoroughly mix additives using a slow swirling action. Slowly add the additives to the media making sure the solution is kept well mixed. Dispense 10mL volumes into sterile tubes to form slopes. Perform a sterility check by incubation at 37°C for 1 week. Store media at 4°C.

## Appendix 2

### Decontamination and Culture Procedures

#### 1. Collection and Choice of Specimens

Collection procedures should avoid contamination of specimens with environmental fungi or bacteria including other mycobacteria. All specimens must reach the laboratory within 48 h of collection. Subsequent laboratory processing of both faecal and tissue samples should occur within 48 h of receipt. Where this is not possible, it is recommended that specimens be stored frozen at -80°C.

##### Individual faeces

At least 2 g of faeces should be collected directly from the rectum and placed in a sterile, leak proof plastic container and refrigerated for transport to the laboratory (using at least a chiller brick in an insulated box).

##### Tissues

The following is a list of tissues devised for cattle that should be collected using aseptic techniques. Each tissue should be divided into two equally representative portions for submission to the laboratory; one refrigerated in a sterile leak-proof container (for culture) and the other in 10% buffered formalin (for histopathology).

Specimens to collect for culture and/or histopathology:

- Entire ileocaecal valve (ICV)
- Ileocaecal lymph nodes
- Ileal (caudal jejunal) lymph nodes
- Two (10 cm) pieces of ileum (one proximal and one distal (terminal))
- One (10cm) piece of proximal colon
- One (10cm) piece of caecum (for histopathology only)

The most commonly affected sites in infected sheep are the terminal ileum and caudal jejunal lymph node.<sup>11, 21</sup>

##### Faeces for pooled faecal culture test

Individual faecal pellets are collected from up to 50 sheep and pooled for culture. Collect one pellet directly from the rectum of each sheep, using a gloved finger, and place in a sterile screw capped jar. Each jar should contain no more than 50 pellets. Change gloves after collection of pellets from each pool.

If a sheep has soft faeces, collect an amount of faeces equivalent to a pellet. Samples jars must be refrigerated prior to shipment to the laboratory. It is necessary to identify the sheep in each pool by ear tags, wool brands or other methods and to record this information on the specimen advice form.

## 2. Sample preparation

### Individual faeces

1.5 to 3 g faeces is mixed with 15 mL saline or water. After 30 min sedimentation, the top 5 mL is transferred to 25 mL HPC/BHI. After incubating at 35–37°C for 16–24 h, the inoculated HPC/BHI is centrifuged at 900 g for 30 min (keep temperature >10°C to avoid precipitation). Alternatively, 2 g of faeces is mixed vigorously in 35 mL, 0.75% HPC/BHI and after incubation as above, 20 mL of the supernatant is transferred (avoiding fibrous sediment) and centrifuged as above. Discard supernatant fluid and resuspend pellet in 1 mL of VAN or VAN/BHI solution. Incubate at 37°C for 24 – 72 h (72 h is recommended to minimise contamination). Inoculate media as described below.

### Tissues

Finely chop 2 g of tissue sample (trimmed of fat) using a sterile scalpel blade or scissors and homogenize or process in a stomacher in 25 mL of 0.75% HPC for 1 min. Allow sample to stand so that foam dissipates and larger pieces of tissue settle. Pour tissue homogenate into a centrifuge tube taking care to avoid carry-over of fat or large tissue pieces. Allow to settle for 30 min then take 10 mL of cellular suspension from just above the sediment to a 30 mL centrifuge tube and incubate at 37°C for 3 h. Centrifuge at 900g for 30 min, discard supernatant fluid and resuspend pellet in 1 mL of VAN or VAN/BHI. Incubate at 37°C overnight. Inoculate media as described below. Alternatively, a sedimentation technique for tissues involves decontamination of homogenized tissue in 0.75% HPC for 24 to 72 h and inoculation of sediment onto media.

### Pooled faeces from small ruminants

Work must be conducted in a manner that minimises the risk of sample-to-sample cross-contamination. Pellets from up to 50 animals are completely homogenized before culture. Samples collected from individual animals on different occasions can also be pooled for culture. The Waring commercial blender base with 250 mL stainless steel blenders are suitable for homogenization.

Any alternative procedure for homogenization needs to ensure adequate mixing and avoid dilution factors that would affect sensitivity.

The procedure for homogenization must ensure there is no sample-to-sample cross-contamination.

Positive and negative faecal controls must be included when processing in each batch of specimens for culture and PCR.

## 3. Culture Methods

### 3.1. Conventional culture for cattle strains of *M paratuberculosis*

For each sample add 0.1 mL to up to 2 or 3 slopes (for tissue and faeces respectively) of Herrold's media containing 2 µg/mL mycobactin J (HEYM) and sodium pyruvate and onto 1 slope of HEYM without sodium pyruvate. Incubate slopes at 37°C for at least 4 months but preferably 6 months.

### 3.2. BACTEC culture for sheep and cattle strains

Inject 0.1 mL of sample (for sheep) or 0.1 to 0.2 mL for other species into one supplemented BACTEC 12B vial. Incubate vials at 37°C for 8 weeks for cattle and 12 weeks for sheep strains. Read BACTEC bottles weekly and record Growth Index (GI).

Further assessment of cultures for mycobactin dependence and/or by PCR testing is required from BACTEC vials having a GI>10 and from slow growing colonies of acid-fast bacilli on solid media.

#### 4. Results and Interpretation

Cultures with no growth:

Cultures with no growth on solid medium or in BACTEC 12B medium after the prescribed incubation period are considered negative.

Conventional culture - solid medium:

Cultures with colonies of typical appearance that are mycobactin dependent are considered consistent with *M. paratuberculosis*. If the colonies are demonstrated to contain IS900 on PCR/REA testing then the sample is considered culture positive for *M. paratuberculosis*.

Radiometric culture - BACTEC 12B medium:

BACTEC cultures with a positive GI that are positive for IS900 on PCR/REA testing are considered to have DNA consistent with *M. paratuberculosis*.

BACTEC cultures may be subcultured to solid medium to examine colony morphology and mycobactin dependency. Isolates with typical colony appearance that are mycobactin dependent are considered consistent with *M. paratuberculosis*. If this sample is also shown to contain IS900 on PCR/REA testing (in either BACTEC or from solid medium) then the sample is considered culture positive for *M. paratuberculosis*.

Where growth in BACTEC medium is identified as being due to organisms other than *M. paratuberculosis* or where overgrowth of irrelevant microbes on solid media renders cultures uninterpretable, the culture is considered to be contaminated. Growth of irrelevant microbes on solid medium does not negate a finding from BACTEC medium of DNA consistent with *M. paratuberculosis*.

Ziehl-Neelsen staining of colonies to demonstrate acid fastness is not obligatory for operators with extensive experience in recognition of typical colonies of *M. paratuberculosis*, but is recommended where there is doubt that colonies are typical of *M. paratuberculosis*. Similarly, the ZN may be applied on BACTEC cultures to confirm the presence of acid fast organisms.

### Appendix 3

#### Ziehl-Neelsen stain

##### 1. Carbol fuchsin

Basic fuchsin	0.3g
Ethanol 95%	10 mL
Phenol	5.0 g
Distilled water	95 mL

##### 2. Acid alcohol

C <sub>2</sub> H <sub>5</sub> OH (95%)	97 mL
Concentrated HCl	3 mL

##### 3. Counterstain

Malachite green or Methylene blue	1.0 g
Distilled water	100 mL

##### 4. Alkali tap water

If malachite green is used as counterstain, it is intensified by washing with alkali tap water prepared by adding 1 mL of 1 M NaOH to 500 mL of tap water.

##### 5. Procedure

Prepare smears and air dry for 10 min. Heat fix at 60 – 70°C for 2 h. Flood each slide with carbol fuchsin. Heat each slide gently until small amount of steam rises. Do not boil. Leave for 5 – 10 min.



Rinse with tap water, then the acid-alcohol for at least 1 – 2 min. Rinse thoroughly with tap water. Flood with counterstain. Leave for 1 – 2 min. Rinse with tap water or alkali tap water. Air dry.

#### 6. Results

Acid-fast bacteria stain red. Other organisms and organic material stain green or blue depending on counterstain.

## Appendix 4

### Mycobactin Dependency

Mycobactin dependency tests for cattle strains can be performed on Herrold's medium or modified 7H10 medium whereas tests on the sheep strain must be performed using modified 7H10 medium.

One colony from the solid media is mixed in 0.5 to 1 mL of PBS. Subculture 0.1 mL volumes of the prepared suspension or BACTEC fluid to slopes of media with and without mycobactin, in each case spreading the inoculum evenly over the surface of the slope and incubate for up to 10 weeks. Alternatively, use a loop to make an even, light inoculum (from a single colony) onto tubes with and without mycobactin.

Acid-fast bacilli which show significantly enhanced growth on media containing mycobactin after at least 2 weeks incubation, with no growth on the unsupplemented slope are considered consistent with *M paratuberculosis*. Acid-fast bacilli not dependent on mycobactin are identified as other mycobacteria (not *M paratuberculosis*).

## Appendix 5

### Polymerase Chain Reaction (PCR)

The PCR developed for the specific detection of *M paratuberculosis* is based on primers that target the insertion sequence IS900.<sup>62</sup> Primers specific for the *Mycobacterium* genus<sup>63</sup> based on the 16S rRNA sequence can also be included in the PCR mix to produce a multiplex PCR that differentiates *M paratuberculosis* from other *Mycobacterium* spp that grow in BACTEC or on solid media.<sup>27</sup>

#### 1. Preparation of Samples

From colonies on solid medium:

A sample of colony growth (normally one colony is sufficient) is mixed in 100 µL of purified sterile water. The suspension is heated at 94-100°C for 30 min (at this stage samples can be stored frozen at -20°C).

From BACTEC vials:

The presence of egg yolk has been found to inhibit the PCR reaction. Consequently when a GI >10 (preferably > 200) is observed, subculture onto solid medium can be performed. BACTEC fluids with a positive GI can also be tested by PCR following either

- (i) subculture into BACTEC without egg yolk; or
- (ii) by removing the egg yolk from the primary culture by alcohol precipitation as follows:

(a) Inoculate 100 µL of the positive sample into BACTEC medium with mycobactin J but without egg yolk or PANTA (although 0.1 mL of PANTA reconstituting fluid is added for its potential to enhance growth). This vial is incubated until the GI is > 200 (about 7 to 14 days). At this stage, 200 µL is removed and heated to 100°C for 30 min ready for testing.

(b) To precipitate egg yolk, 200 µL of culture is transferred to 500 µL absolute ethanol and allowed to stand for 2 min before centrifuging at low speed (8 g for 10 min) to deposit egg yolk on the wall of the tube. The supernatant is centrifuged at high speed (18 000 g for 5 min). The resulting pellet is washed twice in PBS, resuspended in 50 µL sterile purified water and heated at 100°C for 20 min ready for testing.<sup>11, 64</sup> Commercially available ion exchange columns may be used to further reduce inhibition and the PCR repeated if the initial PCR is negative.<sup>21 11</sup>

## 2. PCR Method

A PCR based on the primers described by Vary *et al* (1990)<sup>62</sup> can be used, but other IS900 primers are also suitable.<sup>31, 65</sup> PCR methods should be optimised in each laboratory<sup>66</sup> to determine the optimum concentrations of each of the essential components in the reaction (primers, enzyme, MgCl<sub>2</sub> and nucleotides) and the temperature and time of each of the cycles used in amplification.

As a general guide, primers must be selected from the specific (5') end of IS900, and a minimum annealing temperature of 60°C and 35 cycles of amplification should be used. Usually 5 µL of sample is added to 20 µL of reaction mix. Appropriate laboratory practice should be instituted to prevent contamination.<sup>67</sup> Positive (known *M paratuberculosis* strain) and negative controls (water) must be run in each batch of tests.

5 – 10 µL of PCR product is subjected to electrophoresis in a 2% gel at 80-100 V for 0.6-1 h and stained with 0.5 µg/mL ethidium bromide. Amplified product is visualised using an ultraviolet transilluminator and photographed.

A small number of isolates of mycobacteria other than *M paratuberculosis* have produced an amplified product the same size as that expected from *M paratuberculosis* using both the Vary and Moss/Millar primer systems.<sup>68</sup> These organisms were not mycobactin-dependent and atypical colonial morphology was seen. A restriction enzyme digest must be applied to positive IS900 products to confirm that the amplified sequence is consistent with the sequence of *M paratuberculosis*.

Based on more recent studies, strategies that involve alternate DNA-based testing can be applied to overcome the possibility of a false positive IS900 PCR/REA signal. These include the application of IS1311 PCR and REA, or IS900 sequence analysis. Testing based on these techniques is recommended in samples from flocks or herds where JD has not been previously suspected and/or the likelihood of infection is low, and where results of IS900 PCR and REA yield a conclusion of DNA consistent with *M. paratuberculosis*.

## 3. Results and Interpretation

PCR: The size of the amplified products is estimated after comparison with a 100 base pair molecular weight ladder. The expected size of the IS900 product will depend on the primer sequences selected for use in the PCR reaction. In the multiplex PCR *M paratuberculosis* should produce two amplified products: one consistent with the genus target (1030 bp in size) and the other consistent with the size of the targeted IS900 sequence. Other *Mycobacterium* spp should produce only the genus band in the multiplex PCR.

REA: In the case of the Vary primers, *Hae* III digest results in fragments of 137 and 60 bp from *M paratuberculosis* whereas in the Moss/Millar system, *Mse* I digest produces fragments of 283 and 130 bp. Other enzymes may be used, based on logical choice from DNA sequence data.

## Appendix 6

### Interferon- $\gamma$ Assay (IFN- $\gamma$ )

The commercially available IFN- $\gamma$  kit comprises either 2 or 10 ELISA plates and contains a positive and negative bovine IFN- $\gamma$  ELISA control (BOVIGAM™ CSL Ltd, Parkville Vic 3052). Each plate is pre-coated with anti-bovine IFN- $\gamma$  antibody. Plasma for testing is diluted with supplied diluent, and a HRPO-labelled anti-bovine IFN- $\gamma$  conjugate is used to develop the bound IFN- $\gamma$  with TMB as the substrate. The manufacturer provides instructions for the performance of the assay.

Various interpretations have been used for this assay which is currently undergoing further refinement, particularly in the area of identifying more specific antigens, for use in the diagnosis of Johne's disease.

## Appendix 7

### Agar Gel Immunodiffusion Test (AGID)

#### 1. Antigen

The antigen for this test is prepared from *M paratuberculosis* grown on Watson-Reid medium. Laboratories should use Weybridge strain 316V but it may be preferable to use the goat strain Ama for goats and alpacas. Harvested bacterial cells are suspended in borate or veronal buffer, pH 8.6, and ultrasonically disrupted. Care must be taken to prevent antigens being destroyed by heating during sonication. Alternatively, the cells can be suspended in PBS, pH 7.2, and mechanically disrupted in a French pressure cell. After clarification by centrifugation, the supernatant fluid is titrated against positive sera, and stored in aliquots at -20°C.

Prepared antigens of strains 316V and Ama can be obtained from Graeme Eamens, EMAI, Menangle, NSW (Tel: 046 293 358; Fax: 046 293 384; E-mail: graeme.eamens@agric.nsw.gov.au) or of strain 316V from Geoff de Lisle, AgResearchWallaceville Animal Research Centre, Upper Hutt, NZ (Tel: +64 4 528 6089; Fax: +64 4 528 6605; E-mail: geoffrey.delisle@agresearch.co.nz).

#### 2. Method

Prepare agarose by combining 1.35 g borate (H<sub>3</sub>BO<sub>3</sub>), 0.3 g NaOH, 0.015 g sodium azide and distilled water to 150 mL. Stir to dissolve, adjust pH to 8.5 to 9.0, add Seakem™ agarose to 0.75 – 1.0 % w/v and stand in a boiling water bath until agarose is dissolved. Dispense in appropriate volumes (15 mL for a 90 mm petri dish) and store at 4°C. When required, agarose is melted in a boiling water bath and the contents poured and allowed to set in a 90 mm Petri dish or on a glass slide. Wells are cut in a hexagonal pattern, using a well-cutting stamp. Wells are 4 mm diameter, 2 to 4 mm apart, and 3 to 4 mm deep (volume = 25 to 35  $\mu$ L). Antigen is added to the centre well and test or control sera are added to peripheral wells. It is necessary to include a positive control in every pattern to show the antigen is satisfactory. Any positive reaction is confirmed on a subsequent test with an adjacent control positive. Plates are incubated in a humid chamber at either 37°C overnight or room temperature for 20-24 h.

#### 3. Reading and Interpretation

Greatest specificity is associated with lines that appear within 24 h. The control positive serum should give a strong line halfway (2+ reaction) between the antigen and serum wells. For sheep, this control line may have a slight bend against 316V, unlike goats, which typically show a straight precipitin line between the Ama antigen and the control sera. Lines of non-identity may appear with subsequent incubation, but these are regarded as negative (non-specific reactions). A positive reaction must have a line of identity with the positive control. Lines of partial identity are regarded as suspicious. Precipitin lines are recorded on a scale of 3+ (closer to antigen well than serum well), 2+ (up to half way between the wells) and 1+ (closest to serum well). Control positive sheep and goat sera are available from EMAI, NSW.

## Appendix 8

### Complement Fixation Test (CFT)

#### 1. Sera

The CFT closely follows that for the “Bovine Brucellosis: Serology”. Sera are heated at 58°C (cattle) or 60°C (sheep, goats) for 30 min. If inactivation is performed in plate format in an air incubator, the actual period of incubation must be extended to ensure the sera remain at the required temperature for at least 30 min. A positive control serum should be included on each plate.

#### 2. Antigen

*M avium* strain D4 may be grown on Lowenstein-Jensen, Watson-Reid or other media, harvested, washed and dried. It is recommended that the antigen be ultrasonically disrupted (taking care to contain aerosols during the procedure), rather than autoclaved. One gram of dried cells added to 200 mL distilled water should have a titre of about 1:50 when titrated against positive sera. Prepared antigen is available from the VIAS, Attwood, Victoria, 3049.

#### 3. Complement

Guinea pigs may be bled and the serum preserved in Richardson's buffer, or the complement may be purchased as a lyophilised product from Behring or from Gillies Plains Animal Resource Centre, South Australia. Five C<sub>H</sub>50 are used in the test.

#### 4. Erythrocytes

Washed sheep erythrocytes (3%) are sensitised with approximately three MHD of haemolysin (Behring, Biowhittaker) to give a 1.5% suspension of sensitised cells.

#### 5. Incubation

Primary and secondary incubations are performed at 37°C such that reagents are maintained at the required temperatures for 30 min. In the case of tests performed in plate format, additional time may be required for the plates (and reagents) to reach desired temperatures.

#### 6. Reading and Interpretation

Plates may be left to settle or centrifuged (1500 rpm, 5 min) and read. A reading of 4+ in the 1:8 dilution is regarded as test positive. Positive sera are retested (in duplicate) with the final titre being the mean of at least two consistent results.

The Australian Reference Positive Serum used to calibrate the test is available from VIAS, Attwood, Victoria, 3049.

## Appendix 9

### Absorbed ELISA (Ab-ELISA)

Testing in duplicate is a recommended method for ELISA. Although the Ab-ELISA may be performed in single wells as a screening test it is recommended that samples with reactions +/-10% of the positive cut-off point or greater be retested in duplicate.

The Australian Reference positive and negative antisera for use with the Ab-ELISA for cattle are available from VIAS, Attwood, Victoria.

A summary of the various Ab-ELISA used for testing is shown in the following table:

## ELISA Method

Common and/or commercial name	CSL ELISA/ PARACHEK™ Johne's Absorbed EIA	EMAI ELISA	Agriquality ELISA	Institut Pourquier Bovine Paratuberculosis Test Kit
Developed by	CSL Limited and VIAS, Australia	EMAI, NSW Agriculture, Australia	NZ AgriQuality New Zealand (formally MAF Quality Management)	Institut Pourquier, France
Contact details	CSL Limited, Parkville, VIC, Australia	Microbiology & Immunology Section EMAI, NSW Agriculture Menangle NSW, Australia (Graeme Eamens)	AgriQuality New Zealand, Animal Health Laboratory Palmerston North Institute, NZ (Jo Drake)	Laboratory Diagnostics Pty Ltd. Bankstown NSW Australia
Approved for use in	Cattle, sheep, goats	Cattle, goats, sheep	Cattle, sheep	Cattle
Format	2, 10 and 30 plate commercial kit format	Combination of commercial and in- house reagents	Combination of commercial and in- house reagents	2, 5 and 30 plate commercial kit format
Antigen	<i>M paratuberculosis</i> 316V	<i>M paratuberculosis</i> 316V	<i>M paratuberculosis</i> 316V	<i>M paratuberculosis</i> 316V
<i>M. phlei</i> absorbent (soluble or suspension)	Soluble (30-60 min RT)	Suspension (overnight at 4°C)	Soluble (60 min RT)	Soluble (60 min RT)
Serum dilution	1/20	1/100 (cattle, goats); 1/200 (sheep) in <i>M.</i> <i>phlei</i> solution	1/20	1/20
Conjugate	Mab antibovine/ovine IgG-HRPO	Cattle sera: Mab antibovine/ovine IgG- HRPO  Goat and sheep sera: Polyclonal anticaprine or anti ovine IgG-HRPO	Protein G-HRPO	Mab antibovine IgG- HRPO
Substrate	TMB	ABTS	TMB	TMB
Read	Stop reaction with 0.5M H <sub>2</sub> SO <sub>4</sub> when OD of positive reaches a pre- determined point, then read at 450 nm	Read at 414nm when OD of positive reaches a pre-determined point (cattle, goats), or stop reaction with 0.01% NaN <sub>3</sub> in 0.1M citric acid after 20 min (sheep)	Stop reaction with 0.5M H <sub>2</sub> SO <sub>4</sub> when OD of positive reaches a pre- determined point, then read at 450nm	Stop reaction with 0.5M H <sub>2</sub> SO <sub>4</sub> after 20 min at RT and read at 450 nm
Controls	Positive control sera calibrated against the Australian Reference positive serum to show equivalent reactivity Pos OD between 0.9 and 1.2; Neg<0.15	Pos control (cattle, goats) > 0.5 (preferably 0.55); negative cattle sera typically have OD range 0.05-0.09; negative goat sera OD 0.06-0.1. Cattle test also has phlei and low positive control sera with defined target ELISA ratios.  Sheep serum controls typical ODs: Pos 1.2 – 1.6; Low pos 0.5 – 0.7; Neg 0.15-0.25		Pos control ≥ 0.35