

## **Paratuberculosis (Johne's Disease)**

JM Gwozdz

OIE and National Reference Laboratory for Johne's Disease

Department of Primary Industries

475-485 Mickleham Road,

Attwood, VIC, 3049, Australia

### **Part 1 – Diagnostic overview**

#### **Summary**

*Paratuberculosis (Johne's disease) is a chronic enteritis of ruminants caused by infection with Mycobacterium avium subsp paratuberculosis (M. paratuberculosis), a slow growing, Gram-positive, acid-fast bacillus. There are three strains of this organism; Type I (sheep), Type II (cattle) and Type III (intermittent), which show strong host preference.*

*Young animals are most susceptible to infection. However, most clinical cases occur in adults between 3 and 5 years of age due to a prolonged incubation period. Progressive weight loss and weakness are the main clinical signs in all species. In addition, there is diarrhoea in cattle.*

*Clinically diseased animals may shed billions of M. paratuberculosis organisms per day in faeces. It is assumed that most animals become infected by ingesting the bacilli in manure-contaminated colostrum and milk.*

*Johne's disease is endemic and widespread in sheep, cattle, deer and dairy goats in New Zealand. In Australia, the disease is restricted to parts of temperate south-eastern Australia in dairy and beef cattle, sheep, goats, alpaca, llama and deer. In the affected areas of Australia, cattle, deer, goats and camelids are primarily infected by a cattle strain of M. paratuberculosis, whereas sheep are infected by an ovine strain of M. paratuberculosis. Johne's disease due to infection with an ovine strain of M. paratuberculosis has recently been detected in sheep and a goat in Western Australia.*

*The detection of infection depends on the demonstration of M. paratuberculosis in tissues or faeces by culture or molecular techniques, detection of specific antibodies by serology, or the demonstration of cell-mediated responses. The choice of test depends on the circumstances and the degree of sensitivity required at individual animal or herd level. The single largest problem in Johne's disease control is the difficulty of detecting subclinically infected animals.*

*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, histologically with detection of acid fast organisms, or by detection of M. paratuberculosis using culture or DNA-based techniques.*

*The primary macroscopic lesions of Johne's disease are usually confined to the ileum, caecum, colon and draining lymph nodes and consist of thickening and cording of mesenteric lymphatics, enlargement and oedema of mesenteric lymph nodes, and thickening of the intestinal mucosa as representations of granulomatous lymphangitis, lymphadenitis and enteritis.*

*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 growth. The isolation of M. paratuberculosis from faeces or tissue is the definitive test for Johne's disease. It is the most sensitive diagnostic tool and is considered to be 100% specific.*

*The serological tests commonly used for Johne's disease are absorbed enzyme-linked immunosorbent assay, agarose gel immunodiffusion and complement fixation test. Relative sensitivity and specificity are often determined by reference to results of faecal culture.*

*Tests that measure cell-mediated immune responses, such as a gamma interferon assay or skin test, are not commonly used as diagnostic tools.*

*Safety and containment requirements used in laboratories for common bacterial pathogens apply to work procedures with M. paratuberculosis.*

### Aetiology

Paratuberculosis, or Johne's disease, is a chronic, wasting disease of domestic and wild ruminants caused by infection with *Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis*), formerly known as *M. paratuberculosis* or *M. johnei*.<sup>1-13</sup> It is characterised by granulomatous inflammation of intestines and mesenteric lymph nodes.<sup>14</sup>

*M. paratuberculosis* is a small (0.5 to 1.5 µm), slow growing, Gram-positive, acid-fast bacillus.<sup>1</sup> This organism shares considerable DNA homology with *M. avium* and *M. silvaticum*.<sup>15-16</sup> It was proposed by Thorel et al. (1990) to separate these organisms into 3 distinct clusters: *M. avium* subsp *avium*, *M. avium* subsp *paratuberculosis* and *M. avium* subsp *silvaticum*, subsequent to a large taxonomy study based on phenotypic tests.<sup>17</sup> *M. paratuberculosis* requires supplementation of media with iron-chelated mycobactin for growth *in vitro*.

Several typing methods have been used to investigate the genetic diversity of *M. paratuberculosis*.<sup>18</sup> Based on the patterns obtained by the IS900 restriction fragment length polymorphism (RFLP) and pulsed field gel electrophoresis (PFGE) techniques,

and on growth characteristics and pigmentation, *M. paratuberculosis* isolates are divided into three groups: Type I or sheep (S) strain, Type II or cattle (C) strain and Type III or intermittent strain.<sup>19-20</sup> The single nucleotide polymorphism in the IS1311 sequence assayed by restriction enzyme analysis (REA) of products of the IS1311 polymerase chain reaction (PCR) also distinguishes 'S' (sheep) and 'C' (cattle) strains, which are in intermediate with the Type I (sheep) and Type II (cattle) patterns of the RFLP and PFGE. Although the IS1311 REA does not distinguish between Types I and III, it identifies 'B' (bison) strains within Type II of the RFLP/PFGE classification.<sup>21-22</sup>

There is convincing evidence that these strains show strong host preference. Several studies have demonstrated that regardless of global geographic location, the overwhelming majority of isolates from cattle have been of the Type II or cattle strain. In comparison, most isolates from sheep have produced the Type I (sheep) or Type III (intermediate) strains. Most isolates from goats and deer have belonged to Type II (C).<sup>18-23</sup> A similar, distinct distribution of strains within host populations has been observed in New Zealand and Australia.<sup>19,24-27</sup> Results of a recent study in Australia suggest that based on the current distribution of Johne's disease, the likelihood of transmission of the ovine strain from sheep to cattle is low.<sup>28</sup> The ovine strain has also been found to be less virulent for deer than the bovine type after experimental exposure.<sup>29</sup>

### Clinical Signs

The incubation period of Johne's disease is not well defined. Most clinical cases occur in animals between 3 and 5 years of age.<sup>1,51-52</sup> In all ruminant species, the disease is characterised by gradual weight loss and progressive weakness that advances to lethargy and emaciation.<sup>4,7,9-11,51-55</sup>

In sheep flocks the first indication of the disease may be the development of a distinct 'tail' in the mob. This 'tail' is demonstrated as the weaker animals that drop toward the back of the mob when the animals are mustered. Unlike cattle, where chronic diarrhoea is a typical manifestation of Johne's disease, only a small percentage of clinically affected sheep, goats and deer show diarrhoea, and this is usually confined to the terminal stage of the disease.<sup>4,9,51-53</sup> Similar observations have been reported in llamas and alpacas.<sup>10-11</sup> In affected cattle the faeces are usually green and bubbly and do not contain blood or mucus. Faecal consistency may improve for short periods. The appetite is usually normal, even in animals in an advanced stage of the disease.<sup>55</sup> Wool break has been reported in sheep<sup>56</sup> and deer may show patchy alopecia,<sup>9</sup> presumably due to stress.

A notable feature of Johne's disease in farmed deer is clinical disease in animals as young as 8-12 months of age.<sup>57</sup> Affected animals may have a short illness with weight loss and diarrhoea. Clinical disease is also observed in older deer.<sup>57</sup>

### Epidemiology

Young animals are most susceptible to infection.<sup>30</sup> It is assumed that most animals which become infected, do so shortly after birth.<sup>1</sup> Ingestion of *M. paratuberculosis* is believed to be the primary route of infection.

Clinically diseased animals may shed billions of bacilli per day in faeces. It is assumed that most animals become infected by ingesting the bacilli from a manure-contaminated udder shortly after birth.<sup>1</sup> An oral dose of 1,000 organisms (colony forming units) has caused intestinal infection in lambs and young deer.<sup>3,29</sup> The survival of *M. paratuberculosis* in the environment is favoured by low temperatures, moisture and protection from solar radiation.

The bacteraemia that develops during the course of infection may lead to dissemination of *M. paratuberculosis* to other organs<sup>5,31-33</sup> (including the udder and sex organs) and to vertical transmission of infection.

Intrauterine infection of the foetus has been reported in goats, cattle, sheep and deer.<sup>5,34-37</sup> The percentage of congenitally infected foetuses from cows with clinical Johne's disease ranged from 26.4% to 63.9%,<sup>38</sup> whereas the infection was detected in only 8.6% of foetuses from asymptotically infected cows.<sup>35</sup> A recent study suggests a significant role for bovine intrauterine infection in Johne's disease epidemiology.<sup>39</sup>

In addition, both milk and colostrum may be significant sources of infection, despite the low concentration of *M. paratuberculosis* organisms likely to be present in milk.<sup>40</sup> The organism has been isolated from colostrum of 22% of asymptotically infected cows,<sup>41</sup> from milk of 35% of cows with clinical Johne's disease<sup>42</sup> and from mammary secretions or mammary glands of 3% of clinically affected sheep.<sup>36</sup> Although in cattle, *M. paratuberculosis* excretion via milk appears to be correlated with the stage of infection,<sup>40</sup> the shedding of this organism in bovine faeces and milk is not synchronized as many cows with negative faecal cultures yield positive milk cultures and vice versa.<sup>43</sup>

*M. paratuberculosis* has been recovered from bovine and ovine semen,<sup>44-45</sup> but it is unlikely that venereal transmission plays a significant, if any, role in the epidemiology of Johne's disease.<sup>46</sup>

*M. paratuberculosis* has also been isolated from faeces and tissues of large numbers of wild ruminants and non-ruminant species.<sup>47-50</sup> According to Stevenson *et al.* (2009), the interspecies transmission between wildlife and domestic ruminants occurs, and wildlife may play a role as a reservoir of infection.<sup>50</sup>

### Occurrence and distribution

In Australia, bovine and ovine Johne's disease are restricted to parts of temperate south-eastern Australia in dairy and beef cattle, sheep, goats, deer, and rarely in alpaca and llama. The disease affects animal productivity and market access. Johne's disease is a notifiable disease in all States and the Northern Territory in Australia.

Ovine Johne's disease was first diagnosed in Australia in the central tablelands of NSW in 1980. Further investigations found infected sheep flocks in Victoria, Flinders Island, Kangaroo Island, south-eastern South Australia and mainland Tasmania. The number of infected sheep in a flock varies between 1 and 15% and is typically around 4%. In June 2000 the first case of infection of a goat with the ovine strain of *M. paratuberculosis* was detected in Western Australia followed by the diagnosis of Johne's disease in a number of sheep flocks in that state.

Johne's disease was first recorded in Australian cattle in Warburton, Victoria, in 1925. It is most common in dairy herds, but the disease also occurs in beef cattle, goats, farmed deer and alpacas. At the time of writing, approximately 1,150 cattle herds are officially classified as infected in the south-east of Australia (Victoria, New South Wales, Tasmania and South Australia). The first cases in alpaca and deer were detected in 1993 and 1999, respectively. To prevent the disease from spreading further, zoning for bovine Johne's disease was introduced in 1999. Western Australia has conducted comprehensive testing and is accepted as being a free area for bovine Johne's disease. Endemic infection is not known to occur in Queensland or the Northern Territory. Current estimates of the prevalence of ovine and bovine Johne's disease, and disease management programs adopted in Australia, are available on the Animal Health Australia website.<sup>(1)</sup>

Johne's disease is endemic and widespread in sheep, cattle, deer and dairy goats in New Zealand. The first case shown to be Johne's disease in New Zealand occurred in an imported cow in 1912. Ovine Johne's disease was first reported in New Zealand in South Canterbury in 1952. The first case of Johne's disease was recognised in farmed deer in the late 1980s. Johne's disease is no longer a reportable disease in New Zealand.

### Gross Pathology

It must be noted that many infected animals do not have gross lesions, there is not always a close correlation between the severity of clinical signs and the extent of intestinal lesions, and a wide range of specimens must be examined to ensure a reliable diagnosis.

In cattle, small ruminants and deer the gross pathological findings are similar. Necrosis rarely occurs in cattle and, unlike sheep, goats and deer, there is no caseation or calcification.<sup>14</sup>

Clinically affected animals are usually emaciated, have serous atrophy of fat and effusion in the body cavities. The primary macroscopic lesions of Johne's disease in ruminants are usually confined to the ileum, caecum, colon and draining lymph nodes.<sup>4,8,9,52-54,58-61</sup>

The earliest lesions are thickening and cording of mesenteric lymphatics. In sheep, goats, deer and alpaca the lymphatics may have small, white miliary nodules of caseous necrosis along their length.<sup>9,52,60-62</sup>

The mesenteric and ileocaecal lymph nodes are always enlarged, oedematous and may have focal or diffuse pallor in the cortex. In some goats, sheep and deer, areas of caseous necrosis and mineralisation are present in the cortex and appear as white foci 1-4 mm in diameter.<sup>52,59-62</sup>

The enteric lesions are most common in the terminal ileum and vary from mild, velvety thickening of the mucosa to severe thickening of the bowel with transverse corrugation of the mucosal surface.<sup>8,52,59-62</sup> Necrotic foci in the intestinal mucosa may be found in goats, sheep and deer.<sup>14</sup> In addition, mucosal hyperaemia, erosions and petechiation have been observed in deer.<sup>4,9</sup>

---

(1) [http://www.animalhealthaustralia.com.au/programs/jd/jd\\_home.cfm](http://www.animalhealthaustralia.com.au/programs/jd/jd_home.cfm)

Tubercle-like lesions have also been observed in lymph nodes of the head in deer.<sup>63</sup> Gross changes in deer are very difficult to distinguish from lesions caused by *M. bovis* or other members of the *M. avium* complex.<sup>63-64</sup>

### Diagnostic Tests

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. As such, results of diagnostic tests 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.

Diagnostic sensitivities of tests that detect *M. paratuberculosis*, such as histopathology, culture and DNA probes, are low in animals in early stages of infection but improve as the mycobacterial load increases in advanced stages of infection. A similar association exists between the infection stage and sensitivities of serological tests.

Microscopic examination of Ziehl–Neelsen stained faecal smears lacks sensitivity and specificity and its application is limited to clinical cases to obtain a provisional diagnosis of Johne's disease.

Faecal culture is the best test available for the diagnosis of Johne's disease in live animals. It is believed that culture of faeces on solid media detects about 30–40% of infected cattle. The sensitivity of faecal culture is affected by the stage of infection of the animals tested. It detects only a few animals in the early stages of infection but its sensitivity approaches 100% in clinically affected animals.<sup>72</sup> The isolation of *M. paratuberculosis* from faeces or tissue is considered to be 100% specific.

Histological examination of tissues has been reported to be less sensitive than the conventional culture of tissues in goats and cattle.<sup>60,71</sup>

The enzyme-linked immunosorbent assay (ELISA) is, at present, the most sensitive and specific test for serum antibodies to *M. paratuberculosis* in cattle. It detects about 30–40% of cows identified as infected by culture of faeces on solid media.<sup>72</sup> The complement fixation test (CFT) has been the standard test used for cattle for many years, but it lacks sensitivity and specificity compared to the ELISA. The agar gel immunodiffusion (AGID) test is widely used for testing sheep and goats but not cattle.

Tests that measure cell-mediated immune responses (CMI), such as a gamma interferon assay or skin test, are not commonly used for the diagnosis of Johne's disease in domestic ruminants. The CMI tests tend to detect animals in early stages of infection that produce negative reactions in serological tests.

Although several PCR assays for the direct detection of *M. paratuberculosis* in clinical specimens such as milk or faeces have been reported, the low sensitivity of these tests has limited their adoption.<sup>119-126</sup> The role of the PCR in programs aiming at controlling Johne's disease has primarily been restricted to identification of culture isolates. The PCR has infrequently been used for the confirmation of infection in individual animals by direct detection of *M. paratuberculosis* in tissues submitted for histopathological examination.<sup>127-128</sup> Recent advances in PCR technology and DNA extraction techniques

offer new hope for the development of tests for rapid, sensitive and specific diagnosis of Johne's disease.<sup>129-130</sup> Results of recent studies are promising.<sup>131-133</sup>

In sheep, goats, cattle and alpaca, faecal specimens from several individual animals can be combined and culture undertaken on the pooled sample.<sup>73-75</sup> This approach, known as pooled faecal culture test (PFC), is used as a screening test. The reported herd sensitivity of the PFC in cattle ranges from 22% to almost 100%.<sup>76-81</sup> The specificity of the PFC is considered almost perfect, but it is not uncommon that individual faecal samples from pools tested positive by the PFC yield no growth of *M. paratuberculosis*, presumably due to uneven distribution of the organism in the samples.<sup>76-81</sup>

Culture of environmental samples of manure from areas where a large proportion of cows defecate offers a convenient and unobtrusive alternative strategy to tests currently used to monitor the status of Johne's disease in dairy cattle herds.<sup>82-84</sup> In recent studies in Australia, the culture of 6 replicate samples of manure collected from a milking yard has detected 71% of herds with Johne's disease, whereas testing a singular sample per farm has identified about 40-50% of infected herds.<sup>85-86</sup> This test has a good repeatability and 100% specificity.<sup>86</sup>

Among simulated testing strategies, the culture of environmental samples of manure was the most cost-effective method for detection of Johne's disease in dairy herds followed by PFC, individual faecal culture and serum ELISA with follow-up faecal culture.<sup>87</sup>

### Safety and Containment Requirements

Safety and containment requirements used in laboratories for common bacterial pathogens apply to work procedures with *M. paratuberculosis*.

## Part 2 – Test Methods

### Histopathology (Microscopic lesions)

#### *Principle of the test*

Histological lesions of Johne's disease are characterised by the presence of aggregations of large macrophages with abundant granular cytoplasm, often referred to as epithelioid cells, in the intestinal mucosa and submucosa, lymphatics and in the cortex of mesenteric lymph nodes.<sup>4,8-9,52-54,58-62,65</sup> In the intestines, these aggregations of macrophages are accompanied by focal or diffuse infiltration composed of lymphocytes, eosinophils and occasional neutrophils.<sup>52-54,59,61</sup> Multinucleate giant cells are seen in the intestinal mucosa and cortex of the mesenteric lymph nodes of cattle, deer and small ruminants.<sup>58-59,62</sup>

In some cases, there are focal aggregates of macrophages and scanty acid-fast organisms (AFOs) in the lamina propria. This type of granulomatous inflammatory reaction is frequently classified as 'paucibacillary, tuberculoid', whereas the diffuse infiltration of the intestinal mucosa and submucosa with macrophages that are laden with numerous AFOs is referred to as a 'multibacillary, lepromatous' reaction.<sup>66</sup> The diffuse infiltration of the intestinal mucosa is associated with atrophy of villi and a decrease in the number of crypts.<sup>52</sup>

In some studies, focal areas of caseation and calcification have been observed in the bowel and mesenteric lymph nodes of sheep and goats.<sup>53-54,58-60,67-68</sup> However, other workers have either failed to identify such lesions, or have attributed them to parasitic infestation.<sup>52,61,65,69</sup>

Extensive fibrosis and necrosis in the mesenteric lymph nodes and, in some cases, in lymph nodes of the head is a feature of Johne's disease in deer.<sup>9,63</sup> Identification of the organism by bacterial culture or PCR is required to distinguish lesions in mesenteric lymph nodes and lymph nodes of the head caused by *M. paratuberculosis* from those caused by *M. bovis* and *M. avium*.<sup>63</sup>

Gross lesions in the liver have rarely been reported in sheep,<sup>61</sup> but microgranulomas may be scattered throughout the hepatic parenchyma.<sup>52,61-62</sup> In cattle, aggregates of globule leukocytes have been observed in, or around, myenteric ganglion cells.<sup>62</sup> Lymphocytic neuritis in the gut has been reported in sheep.<sup>70</sup>

In all ruminant species, AFOs are usually numerous within epithelioid cells and multinucleate giant cells in intestinal sections. Fewer AFOs are present in the mesenteric lymph nodes and they are scanty in liver lesions.<sup>52,61-62</sup>

Post-mortem examination of ewes at abattoirs, followed by histopathology of selected tissues, is being used to screen ovine flocks for Johne's disease in Australia.

### *Collection of Specimens*

The following is a list of tissues 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). Samples for histopathology should be stored and shipped at ambient temperature. Storage and shipment conditions of specimens for bacteriology are specified in the 'Bacteriology' section on the next page.

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).

### *Test procedure*

At necropsy, tissues collected into 10% buffered formalin are processed routinely and stained for normal tissue elements with haematoxylin and eosin and also for acid-fast bacilli using the Ziehl-Neelsen method.

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 5 minutes 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 one additional section from each block with lesions is recommended.

### *Quality Control Aspects*

A positive control consisting of tissues with mycobacteria should be included in the Ziehl-Neelsen procedure. The cytoplasm of erythrocytes in tissues properly stained should have a pink tinge.

### *Interpretation of results*

A 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 (in New Zealand the sighting of three acid fast bacilli is required) morphologically consistent with *M. paratuberculosis*.

A finding '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.

## **Bacteriology**

Collection procedures for bacteriological tests should avoid contamination of specimens with environmental fungi or bacteria including other mycobacteria. All specimens must reach the laboratory within 48 hours of collection. Subsequent laboratory processing of both faecal and tissue samples should occur within 48 hours 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).

### *Faeces for pooled faecal culture test (cattle, alpaca, sheep and goats)*

For the pooled faecal culture test, faeces are collected from five cows, or five alpacas, per pool (at least 2 g from each animal) as described above for individual faeces.

Individual faecal pellets are collected from up to 50 sheep or 25 goats and pooled for culture. Collect one pellet directly from the rectum of each animal, using a gloved finger, and place in a sterile screw capped jar. If an animal has soft faeces, collect an amount of faeces equivalent to a pellet.

Change gloves after collection of faeces from each pool. Samples jars must be refrigerated after collection and during shipment to the laboratory. It is necessary to identify animals in each pool by ear tags or other methods and to record this information on the specimen advice form.

## **Microscopy**

### *Principle of the Test*

Examination of Ziehl–Neelsen 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 three or more cells morphologically consistent with *M. paratuberculosis* is consistent with Johne's disease, this is not a definitive test.

### *Reagents and Materials*

See Part 3.

### *Test Procedure*

Prepare smears and air dry for 10 minutes. Heat fix at 60–70°C for 2 hours. Flood each slide with carbol fuchsin. Heat each slide gently until a small amount of steam rises. Do not boil. Leave for 5–10 minutes. Rinse with tap water, then add acid-alcohol for at least 1–2 minutes. Rinse thoroughly with tap water. Flood with counterstain. Leave for 1–2 minutes. Rinse with tap water or alkali tap water and air dry. Examine under immersion oil.

### *Quality Control Aspects*

A positive control (smear containing/spiked with *M. paratuberculosis*) must be included in each batch of specimens for microscopy.

### *Interpretation of Results*

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

## **Culture**

### *Principles of the Test*

The isolation of *M. paratuberculosis* from faeces or tissue is the definitive test for diagnosis of Johne's disease. There are several culture methods, which vary with respect to media, sample type and sample processing protocols. The cultivation of *M. paratuberculosis* is always performed using special media supplemented with mycobactin J.

Since *M. paratuberculosis* organisms are vastly outnumbered by other bacteria or fungi in faecal and intestinal tissue specimens, the successful isolation of the target organism depends on efficient inactivation of the undesirable microbes. However, the decontamination process has a negative effect on *M. paratuberculosis*. Routine decontamination protocols decrease the number of *M. paratuberculosis* organisms by about 2.7 log<sub>10</sub> and 3.1 log<sub>10</sub> for faeces and tissues, respectively.<sup>88</sup> Hexadecylpyridinium chloride (HPC) is recommended as the decontaminant of choice.

There are two techniques for the isolation of *M. paratuberculosis*: (i) conventional culture on solid media and (ii) liquid media culture. The latter method reduces the time required for obtaining a result and is considered to be the more sensitive technique.<sup>89-91</sup> The

decontamination protocol involving double incubation of faecal samples in HPC and a mixture of antibiotics (vancomycin, amphotericin and nalidixic acid) may further improve culture sensitivity.<sup>90</sup> The addition of ampicillin to the media has been reported to reduce the growth of undesired microbes.<sup>92</sup>

Most laboratories in Australia and New Zealand use the liquid media culture method for primary isolation, whereas the conventional culture on solid media is mainly used to determine mycobactin dependency of isolates.

### *Reagents and Materials*

There are several liquid media culture systems. The most commonly used is the radiometric BACTEC 460 culture system (Becton Dickinson), where growth in liquid medium BACTEC™ 12B (Middlebrook 7H12) supplemented with egg yolk and mycobactin is measured by the release of radioactive <sup>14</sup>CO<sub>2</sub>. The BACTEC 460 system is suitable for cultivation of ovine and bovine strains.<sup>90-91</sup> In comparison, the non-radioactive liquid media culture system MGIT 960 (Becton Dickinson) is only suitable for cultivation of bovine strains.<sup>93-94</sup> The incubation time is usually up to 8 and 12 weeks for bovine and ovine strains, respectively. The usefulness of other non-radioactive liquid media culture systems, such as Trek ESPII (Difco) and MB/BacT Alert (Organon Teknika), remains to be determined.

There are two commonly used solid media for the diagnostic isolation of *M. paratuberculosis* in Australia and New Zealand: Herrold's agar (HEY) and modified Middlebrook 7H10. Both media are supplemented with egg yolk and mycobactin. The latter medium supports the growth of both ovine and bovine strains,<sup>91</sup> whereas HEY primarily supports the growth of the bovine strains.<sup>95</sup> It has been reported that the Lowenstein-Jensen (LJ) medium with mycobactin is also suitable for the isolation of both ovine and bovine strains and, in some cases, performs better than 7H10 medium.<sup>95</sup> The addition of sodium pyruvate to HEY may inhibit the growth of some isolates, but in most cases substantially increases the recovery rate and number and size of colonies.<sup>95-98</sup> Other media, such as Dubos medium and Watson-Reid medium, are rarely used.

Primary colonies of *M. paratuberculosis* on solid media may be expected to appear any time from 5 weeks to 6 months after inoculation. The sheep strains grow less well than the cattle strains, and primary cultures on solid media should not be discarded as negative without prolonged incubation, for up to 6-8 months.<sup>95</sup>

Colonies of the cattle strain of *M. paratuberculosis* on HEY medium are typically convex, off-white to cream or buff coloured and non-chromogenic. As cultures age and media dry 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 HEY, 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 HEY, 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 5-7 days. Other organisms may grow on both media with colonies appearing after days or months.

### *Test Procedures*

#### *Decontamination of individual faeces for culture*

Mix 1.5 to 3 g faeces with 15 mL saline or water. After 30 minutes sedimentation, the top 5 mL is transferred to 25 mL 0.9% HPC in half-strength BHI. After incubating at 35–37°C for 16–24 hours, the inoculated HPC in half-strength BHI is centrifuged at 900 *g* for 30 minutes (keep temperature >10°C to avoid precipitation). Alternatively, 2 g of faeces is mixed vigorously in 35 mL 0.75% HPC in half-strength BHI and after incubation, as above, and 20 mL of the supernatant is transferred (avoiding fibrous sediment) and centrifuged as above. Discard the supernatant fluid and resuspend the pellet in 1 mL of VAN or VAN/BHI solution. Incubate at 37°C for 24–72 hours (72 hours is recommended to minimise contamination). Inoculate media as described below.

#### *Decontamination of pooled faeces for culture*

Work must be conducted in a manner that minimises the risk of sample-to-sample cross-contamination. Faecal pellets from up to 50 sheep, 25 goats and faecal samples (2 g each) from five cows or five alpaca 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 and stomachers are suitable for homogenization. The procedure for homogenization must ensure there is no sample-to-sample cross-contamination.

#### *Procedure for sheep*

After homogenization using blenders, samples are processed according to the protocol described above for individual faeces.

The OIE and Australian National Reference Laboratory at Attwood uses stomachers for blending. The submitted faecal sample (pooled pellets from up to 50 individual animals) is weighed, added to a blender bag containing a volume of 0.9% saline equal to 3.5 times the weight of the faeces, homogenised for a minimum of 1 minute, followed by transfer of 10 mL of the mixture to a 25 mL yellow top centrifuge tube containing 10 mL of saline. After mixing by hand the mixture is allowed to settle for 30 minutes, then 5 mL of supernatant is added to a 25 mL tube containing 20 mL of 0.95% HPC in half-strength BHI, mixed, incubated at 37°C for 18–24 hours and centrifuged at 900 *g* for 30 minutes (temperature is kept above 10°C to prevent HPC precipitation). After discarding the supernatant, the pellet is resuspended in 1 mL of VAN or VAN/BHI, incubated at 37°C for 72 hours and 0.1 mL of the resuspended pellet is inoculated using a 1 mL syringe into a vial of Bactec 12B medium supplemented with egg yolk, mycobactin J and PANTA PLUS, and incubated at 37°C for 12 weeks.

*Procedure for goats*

After homogenization using blenders, 1.5-2 g faeces are resuspended in 10 mL saline and, following 30 minutes sedimentation, the top 3.5 mL is transferred to 25 mL 0.9% (w/v) HPC in half strength BHI and incubated at 37°C for 24 hours, followed by centrifugation at 900 g for 30 minutes. After discarding the supernatant, the pellet is resuspended in 1 mL of VAN or VAN/BHI, incubated at 37°C for 72 hours and 0.1 mL of the resuspended pellet is inoculated using a 1 mL syringe into a vial of Bactec 12B medium supplemented with egg yolk, mycobactin J and PANTA PLUS, and incubated at 37°C for at least 10 weeks and up to 12 weeks.

*Procedure for cattle and alpaca*

If using a stomacher system, a volume of sterile saline (50 mL per 10 g faeces) is used for blending. After blending in stomachers, 10 mL of faeces/saline mixture (from 10 g faeces/50 mL saline homogenate) are removed to a screw topped tube. If using blenders, 2 g faeces are resuspended in 10 mL saline prior to homogenisation. The mixture is allowed to settle for 30 minutes and the top 3.5 to 5 mL are transferred to 25 mL 0.9% HPC in half-strength BHI and then processed as described for pooled faeces from goats.

*Decontamination of tissues for culture*

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 minute. Allow the 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 minutes, then take 10 mL of cellular suspension from just above the sediment into a 30 mL centrifuge tube and incubate at 37°C for 3 hours. Centrifuge at 900 g for 30 minutes, discard the supernatant fluid and resuspend the 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 hours and inoculation of sediment onto media.

*Inoculation and incubation of conventional cultures for cattle strains*

For each decontaminated sample add 0.1 mL to up to 2 or 3 slopes (for tissue and faeces respectively) of HEY supplemented with 2 µg/mL mycobactin J (HEYM) and sodium pyruvate and onto one slope of HEYM without sodium pyruvate. Incubate slopes at 37°C for at least 4 months but preferably 6 months.

*Inoculation and incubation of BACTEC cultures for sheep and cattle strains*

Inject 0.1 mL of decontaminated 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 strains 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.

*Quality Control Aspects*

Negative and positive (faeces spiked with *M. paratuberculosis*) controls must be included in each batch of specimens for culture.

*Interpretation of Results*

*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 may be subcultured to a 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'.

BACTEC cultures with a positive GI that are positive for IS900 on PCR/REA testing are considered to have 'DNA consistent with *M. paratuberculosis*'. Growth of irrelevant microbes on solid medium does not negate a finding from BACTEC medium of DNA consistent with *M. paratuberculosis*.

**Isolate identification**

*Principle of the Test*

The identification of *M. paratuberculosis* isolates commonly relies on the slow growth rate of acid-fast bacilli, demonstration of mycobactin dependency and detection of IS900, a DNA fragment that is considered to be unique for the *M. paratuberculosis* genome.<sup>15-16</sup>

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 Ziehl-Neelsen stain may be applied on BACTEC cultures to confirm the presence of acid fast organisms.

For the demonstration of mycobactin dependency, small inoculum of suspect colonies should be subcultured on the same solid medium with and without mycobactin. Mycobactin is present in the cell wall of the organism, and heavy inoculum may contain enough of this compound to support the growth of *M. paratuberculosis* on the medium without mycobactin, leading to misidentifications and false negative results.

In addition, the identification process may be confounded by infections caused by mycobactin-dependent *M. sylvaticum* and *M. avium* strains,<sup>99-101</sup> the difficulty in the isolation of some ovine strains of *M. paratuberculosis*,<sup>52,102-105</sup> variations in mycobactin-dependence of the organism under different culture conditions,<sup>106</sup> and presence of IS900 in mycobacteria other than *M. paratuberculosis*.

Since the discovery of the IS900 by Green et al. (1989),<sup>107</sup> the detection of this DNA element by the PCR has been reported in six unidentified, mycobactin-independent mycobacterial isolates, of which one was related to *M. cookie*,<sup>108</sup> three were related to *M. scrofulaceum*,<sup>109</sup> one belonged to MAIC<sup>110</sup> and one was not investigated further.<sup>111</sup> In addition, Herthnek and Bölske (2006) have reported detection of IS900 by various real-time PCR systems in two unidentified, mycobactin-independent mycobacterial isolates and in one *M. avium* isolate.<sup>112</sup>

Although the presence of IS900-like sequences in mycobacteria other than *M. paratuberculosis* is extremely rare, the identification of new DNA fragments considered unique to this organism (ISMav2, f57, hspX and ISMap02 sequences), offers additional tools for rapid identification of this organism using the PCR.<sup>113-116</sup> However, the PCR tests based on the new sequences may have reduced analytical sensitivity due to lower numbers of target DNA in the *M. paratuberculosis* genome compared to the IS900.<sup>115-118</sup>

The difficulties in cultivation of the sheep strains of *M. paratuberculosis* are circumvented by the use of the liquid (BACTEC 12B) medium and solid medium based on the Middlebrook 7H10 formulation.<sup>78</sup>

### *Reagents and Materials*

#### *Mycobactin Dependency*

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

#### *PCR*

For the identification of culture-derived isolates, a PCR test based on primers that target the insertion sequence IS900<sup>157-158</sup> can be used, but other IS900 primers are also suitable.<sup>122</sup> Primers specific for the *Mycobacterium* genus 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>161</sup>

### *Test Procedures*

#### *Mycobactin Dependency*

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.

*PCR*

*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 minutes (at this stage samples can be stored frozen at -20°C).

*Preparation of samples 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 7-14 days). At this stage, 200 µL is removed and heated to 100°C for 30 minutes to make 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 minutes before centrifuging at low speed (8 g for 10 minutes) to deposit egg yolk on the wall of the tube. The supernatant is centrifuged at high speed (18,000 g for 5 minutes). The resulting pellet is washed twice in PBS, resuspended in 50 µL sterile purified water and heated at 100°C for 20 minutes to make ready for testing.<sup>91</sup> Commercially available ion exchange columns may be used to further reduce inhibition and the PCR repeated if the initial PCR is negative.

*PCR 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. All PCR methods, both conventional and real-time variants, should be validated and optimised in each laboratory 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.<sup>159</sup> Appropriate laboratory practice should be instituted to prevent contamination.<sup>160</sup>

*Quality Control Aspects*

Positive (known *M. paratuberculosis* strain) and negative controls (water) must be run in each batch of PCR tests.

If there is a doubt about the validity of the PCR result, a restriction enzyme digest may be applied to positive IS900 products derived from the conventional PCR to confirm that the amplified sequence is consistent with the sequence of *M. paratuberculosis*. Alternatively, the IS1311 PCR and REA could be applied, the IS900 PCR product may be sequenced, or the isolate tested for other DNA fragments that are considered unique for *M. paratuberculosis*.<sup>113-116</sup> Testing based on the techniques listed above is recommended in samples from flocks or herds where Johne's disease 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*.

### *Interpretation of Results*

#### *Mycobactin Dependency*

Acid-fast bacilli that 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*).

#### *PCR*

For the conventional PCR systems, 5–10 µL of PCR product is subjected to electrophoresis in a 2% gel at 80-100 V for 0.6-1 hour and stained with 0.5 µg/mL ethidium bromide. Amplified product is visualised using an ultraviolet transilluminator and photographed. 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.<sup>161</sup> Other *Mycobacterium* spp should produce only the genus band in the multiplex PCR.

In the case of the Vary primers, the REA using *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 130bp. Other enzymes may be used, based on logical choice from DNA sequence data.

For the real-time PCR systems, a curve of each sample needs to be visually examined for the presence of exponential and plateau phases before results are classified as positive based on the pre-determined cut-off values.

### **Immunological tests**

There is a prolonged delay between infection with *M. paratuberculosis* and detection of the systemic immune response. The detection of a systemic cell-mediated immunity (CMI) response precedes detectable antibody production. Animals that are minimally infected frequently react positively to tests that measure CMI, but do not react on serological testing. In contrast, some sero-positive animals have no detectable CMI response. It is believed that the CMI is inversely related to antibody response as the disease progresses. Serum antibodies are present more constantly and are of higher titre as lesions become more extensive, reflecting the amount of antigen present. In advanced stages of infection animals may become anergic and have no detectable antibody or CMI responses.

### **Enzyme-linked immunosorbent assay**

#### *Principle of the Test*

The sensitivity of ELISA is comparable with that of the complement fixation test (CFT) in clinical cases, but is greater than that of the CFT in subclinically infected carriers. The specificity of the ELISA is increased by *M. phlei* absorption of sera.<sup>134</sup>

### *Reagents and Materials*

Three absorbed ELISA kits are currently approved for use in cattle in Australia, of which one is also approved for use in sheep and goats (see Part 3). On-going evaluations of these commercial kits by the Australian National Johne's Disease Reference Laboratory have so far demonstrated that they have similar diagnostic performance. An additional absorbed ELISA is in use in New Zealand. A non-absorbed ELISA has also been used in deer in New Zealand, but this test had not been approved by SCAHLS at the time of writing.<sup>137</sup>

### *Quality Control Aspects*

Testing in duplicate is a recommended method for ELISA. Although the ELISA may be performed in single wells as a screening test it is recommended that samples with reactions within +/-10% of the positive cut-off point be retested in duplicate. Positive and negative control sera are included in each run.

### *Interpretation of Results*

The sensitivity of the ELISA depends on the age of animal, stage of infection and the degree of *M. paratuberculosis* shedding in faeces. In a large study in Australia, the actual sensitivity of the ELISA in 2-, 3- and 4-year-old cows was 1.2%, 8.9% and 11.6%, respectively, but remained between 20 and 30% in older age-groups.<sup>135</sup> The overall sensitivity for all infection stages and age-groups was calculated to be about 15%.<sup>72,135</sup> The three commercial ELISA kits available in Australia have specificities above 99%.

In small ruminants, the commercially available ELISA had a specificity of 98.2–99.5% and detected 35–54% of animals with histological evidence of infection.<sup>136</sup>

Some commercial kits offer an option of testing milk samples. The ELISA on bovine and caprine milk has been found to have a specificity similar to that of the serum ELISA, but is less sensitive than the blood test.<sup>138-139</sup>

## **Agar gel immunodiffusion test (AGID)**

### *Principle of the Test*

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 and requires subjective interpretation. It has been reported that in small ruminants in New Zealand and Australia the AGID offers slightly higher sensitivity and specificity than that obtained by the ELISA tests.<sup>136,140-141</sup> The reported specificity and sensitivity of the AGID measured against histological results were 99-100% (95%CI) and 38-56% (95% CI), respectively.<sup>136</sup>

### *Reagents and Materials*

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 (see Part 3). 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.

### *Test Procedure*

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 8.5-9.0, 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, 2-4 mm apart, and 3-4 mm deep (volume = 25 to 35 µL). 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 that 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 at room temperature for 20-24 hours.

### *Quality Control Aspects*

Positive and negative control sera are included in each run.

### *Interpretation of Results*

Greatest specificity is associated with lines that appear within 24 hours. 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).

## **Complement fixation test**

### *Principle of the Test*

The CFT works well on clinically suspect animals, but does not have sufficient sensitivity and specificity to enable its use in the general population for disease control, diagnosis or certification purposes. Nevertheless, it is often demanded by countries that import cattle from Australia and New Zealand.

### *Reagents and Materials*

The test is performed using *M. avium* strain D4 (see Part 3), which is more readily cultivated and gives similar reactivity to antigens prepared from *M. paratuberculosis*.<sup>142</sup>

The CFT closely follows that for the “Bovine Brucellosis: Serology” as described in the Australian Standard Diagnostic Techniques for Animal Diseases (1993), Ed. LA Corner ([http://www.scahls.org.au/data/assets/pdf\\_file/0007/1280824/bovine\\_brucellosis.pdf](http://www.scahls.org.au/data/assets/pdf_file/0007/1280824/bovine_brucellosis.pdf)).

Sera are heated at 58°C (cattle) or 60°C (sheep and goats) for 30 minutes. 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 minutes. A positive control serum (see Part 3) should be included on each plate. Guinea pigs may be bled and the serum preserved in Richardson's buffer, or the complement may be purchased as a lyophilised product from Dade Behring Diagnostics. Five complement haemolytic activity units ( $CH_{50}$ ) are used in the test. Washed sheep erythrocytes (3%) are sensitised with approximately three minimal haemolytic doses (MHD) of haemolysin (Dade Behring Diagnostics) to give a 1.5% suspension of sensitised cells.

### *Test Procedure*

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

### *Quality Control Aspects*

Positive and negative control sera are included in each run.

### *Interpretation of Results*

Plates may be left to settle or centrifuged (1,500 rpm for 5 minutes) 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.

## **Tests for cell-mediated immunity**

These include the gamma interferon assay and the intradermal johnin test. Other tests have been used to assess CMI responses but have never been widely accepted as diagnostic tools. These tests include the intravenous johnin test, lymphocyte transformation/stimulation/proliferation tests and the leucocyte migration inhibition test.<sup>150-156</sup>

## **Gamma interferon assay**

### *Principle of the Test*

The gamma interferon assay (BOVIGAM) was developed for the diagnosis of bovine tuberculosis. It measures the release of gamma interferon from sensitised lymphocytes during an 18–36 hour incubation period with specific antigen (avian tuberculin purified protein derivative [PPD], bovine tuberculin PPD or johnin PPD).<sup>143</sup> The test procedure and materials are fully described in the instructions accompanying the commercial kit. This test has not been validated by the manufacturer (Prionics, Switzerland) for the diagnosis of Johne's disease. As such, results derived from this assay are frequently difficult to interpret because there is no agreement with respect to the interpretation criteria and types and amounts of antigens used to stimulate blood lymphocytes.

### *Quality Control Aspects*

Positive and negative control sera are included in each run. Results are validated following the manufacturer's recommendations.

### *Interpretation of Results*

In cattle, the reported specificity of the gamma interferon assay varied from 94% to 67% depending on the interpretation criteria.<sup>144</sup> In sheep the reported sensitivity of this test was 66.7% in tissue culture-positive animals, and the specificity was 98.3% in non-infected flocks.<sup>145</sup> The gamma interferon assay is unreliable in calves under 12 months of age due to the occurrence of many false positive results.<sup>146-148</sup>

### **Johnin skin test**

#### *Principle of the Test*

The skin test for delayed-type hypersensitivity (DTH) measures CMI, but has limited value. The test is still required by some overseas countries for certification of imported cattle and sheep.

#### *Reagents and Materials*

The test is conducted in the same manner as the tuberculin test using johnin PPD or *M. avium* PPD (25,000 units/mL, AsureQuality, New Zealand). Johnin PPD is not currently available from CSL and is unlikely to be available in the future. If the johnin skin test is mandatorily required by the importing country, then johnin PPD should be sourced from that country (or the country provide a source) subject to approval from Australia's regulatory authorities.

#### *Test Procedure*

The test is conducted by intradermal inoculation of 0.1 mL johnin PPD into the caudal fold or the mid-neck region. The skin thickness is measured with calipers before and 72 hours after inoculation; an increase in skin thickness of >3 mm being taken as positive.

#### *Interpretation of Results*

It should be noted that positive reactions in deer may take the form of diffuse plaques rather than discrete circumscribed swellings, thus making reading of the test more difficult. The presence of any swelling should be regarded as positive in this species. However, sensitisation to the *M. avium* complex is widespread in animals, and neither avian tuberculin nor johnin are highly specific.<sup>155</sup> Furthermore, the interpretation of the skin test results is complicated by the lack of agreement with respect to interpretation criteria.

In a recent study in which johnin (ID-Lelystad, The Netherlands) was used to test cattle, the skin test specificity was 88.8% at the cut-off value of  $\geq 2$  mm, 91.3% at the cut-off value of  $\geq 3$  mm and 93.5% at the cut-off value of  $\geq 4$  mm.<sup>144</sup> The effect of these cut-off values on the sensitivity has not been determined. The performance of this test may also be significantly affected by minor antigenic differences that occur in different batches of antigen.<sup>144</sup>

### **Acknowledgements**

The 1993 version of this section was written by L. Stephens. A workshop was sponsored in 1995 by the Dairy Research and Development Corporation and the Meat Research Corporation to promote standardisation of diagnostic methods. A further workshop in

1999, funded through the National Ovine JD Control and Evaluation Program, concentrated on bacteriological culture, including pooled faecal culture and identification of sheep strains. These workshops resulted in production of revised sections in 1998-2000 by JM Tennent, DV Cousins, RJ Condrón, GJ Eamens and RJ Whittington.

The current edition incorporates advances in knowledge, technology and standardisation since the last revision performed in 2002 by DV Cousins, RJ Condrón, GJ Eamens, RJ Whittington and GW de Lisle.

### References

1. Chiodini RJ, Van Kruiningen HJ, Merkal RS. Ruminant paratuberculosis (Johne's disease): The current status and future prospects. *Cornell Vet* 1984;74:218-262.
2. Bang B. Chronische pseudotuberculose Darmentzündung beim Rinde. *Berl. Munch. Tierarztl. Wochenschr* 1906;42:759-763.
3. Brotherston JG, Gilmour NJL, McSamuel J. Quantitative studies of *Mycobacterium johnei* in the tissues of sheep. I. Routes of infection and assay of viable *M. johnei*. *J Comp Pathol* 1961;71:286-299.
4. Vance HN. Johne's disease in a European red deer. *Can Vet J* 1961;2:305-307.
5. Goudswaard J. Studies on the incidence of *Mycobacterium johnei* in the organs of experimentally infected goats. *Neth J Vet Sci* 1971;4:65-75.
6. Boever WJ. Johne's disease in aoudads and mouflon. *J Zoo Animal Med* 1976;7:19-23.
7. Williams ES, Snyder SP, Martin KL. Experimental infection of some North American wild ruminants and domestic sheep with *Mycobacterium paratuberculosis*: clinical and bacteriological findings. *J Wildl Dis* 1983;3:185-191.
8. Williams ES, Snyder SP, Martin KL. Pathology of spontaneous and experimental infection of North American wild ruminants with *Mycobacterium paratuberculosis*. *Vet Pathol* 1983;20:274-291.
9. Gilmour N, Nyange J. Paratuberculosis (Johne's disease) in deer. *In Practice* 1989;11:193-196.
10. Belknap EB, Getzy DM, Johnson LW, Ellis RP, Thompson GL, Shulaw WP. *Mycobacterium paratuberculosis* infection in two llamas. *J Am Vet Assoc* 1994;204:1805-1808.
11. Ridge S E, Harkin J T, Badman R T, Mellor A M, Larsen J W A. Johne's disease in alpacas (*Lama pacos*) in Australia. *Aust Vet J* 1995;72:150-153.
12. Machackova M, Svastova P, Lamka J, Parmova I, Liska V, Smolik J, Fischer OA, Pavlik I. Paratuberculosis in farmed and free-living wild ruminants in the Czech Republic (1999-2001). *Vet Microbiol* 2004;101:225-34.
13. Crawford GC, Ziccardi MH, Gonzales BJ, Woods LM, Fischer JK, Manning EJ, Mazet JA. *Mycobacterium avium* subspecies *paratuberculosis* and *Mycobacterium*

- avium* subsp. *avium* infections in a tule elk (*Cervus elaphus nannodes*) herd. *J Wildl Dis* 2006;42:715-23.
14. Brown CC, Baker DC, Barker IK. Infectious and parasitic diseases of the gastrointestinal tract. In: Maxie MG, editor. *Jubb, Kennedy, and Palmer's Pathology of Domestic Animals*. 5th edn. 3 vols, Elsevier, 2007;2:222-225.
  15. McFadden JJ, Butcher PD, Chiodini R, Hermon-Taylor J. Crohn's disease-isolated mycobacteria are identical to *Mycobacterium paratuberculosis*, as determined by DNA probes that distinguish between mycobacterial species. *J Clin Microbiol* 1987;25:796-801.
  16. McFadden JJ, Butcher PD, Thompson J, Chiodini R, Hermon-Taylor J. The use of DNA probes identifying restriction-fragment length polymorphisms to examine the *Mycobacterium avium* complex. *Mol Microbiol* 1987;1:283-291.
  17. Thorel MF, Krichevsky M, Vincent Levy-Frebault V. Numerical taxonomy of mycobactin-dependent mycobacteria, emended description of *Mycobacterium avium*, and description of *Mycobacterium avium* subsp. *avium* subsp. nov., *Mycobacterium avium* subsp. *paratuberculosis* subsp. nov., and *Mycobacterium avium* subsp. *silvaticum* subsp. nov. *Int J Syst Bacteriol*. 1990;40:254-260.
  18. Motiwala AS, Li L, Kapur V, Sreevatsan S. Current understanding of the genetic diversity of *Mycobacterium avium* subsp. *paratuberculosis*. *Microbes Infect* 2006;8:1406-18.
  19. Collins DM, Gabric DM, De Lisle GW. Identification of two groups of *Mycobacterium a. paratuberculosis* strains by restriction endonuclease analysis and DNA hybridisation. *J Clin Microbiol* 1990;28:1591-1596.
  20. de Lisle GW, Collins DM, Huchzermeyer HK. Characterization of ovine strains of *Mycobacterium. paratuberculosis* by restriction endonuclease analysis and DNA hybridization. *Onderstepoort J Vet Res* 1992;59:163-165.
  21. Marsh I, Whittington R, Cousins D. PCR-restriction endonuclease analysis for identification and strain typing of *Mycobacterium avium* subsp *paratuberculosis* and *Mycobacterium avium* subsp *avium* based on polymorphisms in IS1311. *Mol Cell Probes* 1999;13:115-126.
  22. Whittington RJ, Marsh IB, Whitlock RH: Typing of IS1311 polymorphisms confirms that bison (*Bison bison*) with paratuberculosis in Montana are infected with a strain of *Mycobacterium avium* subsp. *paratuberculosis* distinct from that occurring in cattle and other domesticated livestock. *Mol Cell Probes* 2001;15:139-145.
  23. Sevilla I, Garrido JM, Geijo M, Juste RA. Pulsed-field gel electrophoresis profile homogeneity of *Mycobacterium avium* subsp. *paratuberculosis* isolates from cattle and heterogeneity of those from sheep and goats. *BMC Microbiol*. 2007;12:7-18.
  24. Cousins DV, Williams SN, Hope A, Eamens G. DNA fingerprinting of Australian isolates of *Mycobacterium avium* subsp *paratuberculosis* using IS900 RFLP. *Aust Vet J* 2000;78:184-190.

25. Whittington RJ, Hope AF, Marshall DJ, Taragel CA, Marsh I. Molecular epidemiology of *Mycobacterium avium* subsp. *paratuberculosis*: IS900 restriction fragment length polymorphism and IS1311 polymorphism analyses of isolates from animals and a human in Australia. *J Clin Microbiol* 2000;38:3240-3248.
26. Whittington RJ, Tarragel CA, Ottaway S, Marsh I, Seaman J, Fridriksdottir V. Molecular epidemiological confirmation and circumstances of occurrence of sheep (S) strains of *Mycobacterium avium* subsp. *paratuberculosis* in cases of paratuberculosis in cattle in Australia and sheep and cattle in Iceland. *Vet Microbiol* 2001;79:311-322.
27. de Lisle GW, Cannon MC, Yates GF, Collins DM. Use of a polymerase chain reaction to subtype *Mycobacterium avium* subspecies *paratuberculosis*, an increasingly important pathogen from farmed deer in New Zealand. *N Z Vet J* 2006;54:195-7.
28. Moloney BJ, Whittington RJ. Cross species transmission of ovine Johne's disease from sheep to cattle: an estimate of prevalence in exposed susceptible cattle. *Aust Vet J* 2008;86:117-23.
29. Mackintosh CG, Labes RE, Clark RG, de Lisle GW, Griffin JF. Experimental infections in young red deer (*Cervus elaphus*) with a bovine and an ovine strain of *Mycobacterium avium* subsp *paratuberculosis*. *N Z Vet J* 2007;55:23-9.
30. Payne JM, Rankin JD. Comparison of the pathogenesis of experimental Johne's disease in calves and cows. *Res Vet Sci* 1961;2:175-179.
31. Hines SA, Buergelt CD, Wilson JH, Bliss EL. Disseminated *Mycobacterium paratuberculosis* infection in a cow. *J Am Vet Med Assoc* 1987;190:681-683.
32. Rohde RF, Shulaw WP. Isolation of *Mycobacterium paratuberculosis* from the uterine flush fluids of cows with clinical paratuberculosis. *J Am Vet Med Assoc* 1990;197:1482-1483.
33. Koenig GJ, Hoffsis GF, Shulaw WP, Bech-Nielsen S, Rings DM, St-Jean G. Isolation of *Mycobacterium paratuberculosis* from mononuclear cells in tissues, blood and mammary glands of cows with advanced paratuberculosis. *Am J Vet Res* 1993;54:1441-1445.
34. Seitz SE, Heider LE, Hueston WD, Bech-Nielsen S, Rings DM, Spangler L. Bovine fetal infection with *Mycobacterium paratuberculosis*. *J Am Vet Med Assoc* 1989;194:1423-1426.
35. Sweeney RW, Whitlock RH, Rosenberger AE. *Mycobacterium paratuberculosis* isolated from fetuses of infected cows not manifesting signs of the disease. *Am J Vet Res* 1992;53:477-480.
36. Lambeth C, Reddacliff LA, Windsor P, Abbott KA, McGregor H, Whittington RJ. Intrauterine and transmammary transmission of *Mycobacterium avium* subsp *paratuberculosis* in sheep. *Aust Vet J* 2004;82:504-8.
37. Intra-uterine transmission of *Mycobacterium avium* subsp *paratuberculosis* in subclinically affected red deer (*Cervus elaphus*). Thompson BR, Clark RG,



- Mackintosh CG. *N Z Vet J* 2007;55:308-13.
38. Gay JM, Sherman DM. Factors in the epidemiology and control of ruminant paratuberculosis. *Vet Med* 1992;1133-1139.
  39. Whittington RJ, Windsor PA. In utero infection of cattle with *Mycobacterium avium* subsp. *paratuberculosis*: a critical review and meta-analysis. *Vet J* 2009;179:60-9.
  40. Sweeney RW, Whitlock RH, Rosenberger AE. *Mycobacterium paratuberculosis* cultured from milk and supramammary lymph nodes of infected asymptomatic cows. *J Clin Microbiol* 1992;30:166-171.
  41. Streeter RN, Hoffsis GF, Bech-Nielsen S, Shulaw WP, Rings DM. Isolation of *Mycobacterium paratuberculosis* from colostrum and milk of subclinically infected cows. *Am J Vet Res* 1995;56:1322-1324.
  42. Taylor TK, Wilks CR, McQueen DS. Isolation of *Mycobacterium paratuberculosis* from the milk of cows with Johne's disease. *Vet Rec* 1981;109:532-533.
  43. Gao A, Odumeru J, Raymond M, Hendrick S, Duffield T, Mutharia L. Comparison of milk culture, direct and nested polymerase chain reaction (PCR) with fecal culture based on samples from dairy herds infected with *Mycobacterium avium* subsp. *paratuberculosis*. *Can J Vet Res* 2009;73:58-64.
  44. Larsen AB, Kopecky KE. *Mycobacterium paratuberculosis* in reproductive organs and semen of bulls. *Am J Vet Res* 1970;31:255-258.
  45. Eppleston J, Whittington RJ. Isolation of *Mycobacterium avium* subsp. *paratuberculosis* from the semen of rams with clinical Johne's disease. *Aust Vet J* 2001;79:776-7.
  46. Merkal RS, Miller JM, Hintz A.M, Bryner JH. Intrauterine inoculation of *Mycobacterium paratuberculosis* into guinea pigs and cattle. *Am J Vet Res* 1982;43:676-678.
  47. Beard PM, Daniels MJ, Henderson D, Pirie A, Rudge K, Buxton D, Rhind S, Greig A, Hutchings MR, McKendrick I, Stevenson K, Sharp JM. Paratuberculosis infection of nonruminant wildlife in Scotland. *J Clin Microbiol* 2001;39:1517-21.
  48. Greig A, Stevenson K, Perez V, Pirie AA, Grant JM, Sharp JM. Paratuberculosis in wild rabbits (*Oryctolagus cuniculus*). *Vet Rec* 1997;140:141.
  49. McClure HM, Chiodini RJ, Anderson DC, Swenson RB, Thayer WR, Coutu JA. *Mycobacterium paratuberculosis* infection in a colony of stump-tail macaques (*Macaca arctoides*). *J Inf Dis* 1987;155:1011.
  50. Stevenson K, Alvarez J, Bakker D, Biet F, de Juan L, Denham S, Dimareli Z, Dohmann K, Gerlach GF, Heron I, Kopečna M, May L, Pavlik I, Sharp JM, Thibault VC, Willemsen P, Zadoks RN, Greig A. Occurrence of *Mycobacterium avium* subspecies *paratuberculosis* across host species and European countries with evidence for transmission between wildlife and domestic ruminants. *BMC Microbiol* 2009;9:212.

51. Gezon HM, Bither HD, Gibbs HC, Acker EJ, Hanson LA, Thompson JK, Jorgenson RD. Identification and control of paratuberculosis in a large goat herd. *Am J Vet Res* 1988;49:1817-1823.
52. Carrigan MJ, Seaman JT. The pathology of Johne's disease in sheep. *Aust Vet J* 1990;67:47-50.
53. Morin M. Johne's disease (paratuberculosis) in goats: A report of eight cases in Quebec. *Can Vet J* 1982;23:55-58.
54. Thomas GW. Paratuberculosis in a large goat herd. *Vet Rec* 1983;113:464-466.
55. Whitlock RH, Buergelt C. Preclinical and clinical manifestations of paratuberculosis (including pathology). *Vet Clin North Am Food Anim Pract* 1996;12:345-356.
56. Cranwell, M.P. Control of Johne's disease in a flock of sheep by vaccination. *Vet Rec* 1993;133:219-220.
57. Mackintosh CG, de Lisle GW, Collins DM, Griffin JF. Mycobacterial diseases of deer. *N Z Vet J* 2004;52:163-74.
58. Stamp JT, Watt JA. Johne's disease in sheep. *J Comp Pathol* 1954;64:26-40.
59. Rajya BS, Singh CM. Studies on the pathology of Johne's disease in sheep. III. Pathologic changes in sheep with naturally occurring infection. *Am J Vet Res* 1961;22:189-203.
60. Fodstad FH, Gunnarsson E. Post-mortem examination in the diagnosis of Johne's disease in goats. *Acta Vet Scand* 1979;20:157-167.
61. Clarke CJ, Little D. The pathology of ovine paratuberculosis: Gross and histological changes in the intestine and other tissues. *J Comp Pathol* 1996;111:419-437.
62. Buergelt CD, Hall C, McEntee K, Duncan JR. Pathological evaluation of paratuberculosis in naturally infected cattle. *Vet Pathol.* 1978;15:196-207.
63. de Lisle GW, Yates GF, Montgomery H. The emergence of *Mycobacterium paratuberculosis* in farmed deer in New Zealand - a review of 619 cases. *N Z Vet J* 2003;51:58-62.
64. Glawischnig W, Steineck T, Spargser J. Infections caused by *Mycobacterium avium* subspecies *avium*, *hominissuis*, and *paratuberculosis* in free-ranging red deer (*Cervus elaphus hippelaphus*) in Austria, 2001-2004. *J Wildl Dis* 2006;42:724-31.
65. Perez V, Garcia Marin JF, Badiola JJ. Description and classification of different type of lesions associated with natural paratuberculosis infection in sheep. *J Comp Pathol* 1996;114:107-122.
66. Clarke CJ. The pathology and pathogenesis of paratuberculosis in ruminants and other species. *J Comp Pathol* 1997;116:217-261.
67. Juste RA, Garcia Marin JF, Peris B, Saez de Ocariz C, Badiola JJ. Experimental infection of vaccinated and non-vaccinated lambs with *Mycobacterium paratuberculosis*. *J Comp Pathol* 1994;110:185-194.

68. Tafti AK, Rashidi K. The pathology of goat paratuberculosis: gross and histopathological lesions in the intestines and mesenteric lymph nodes. *J Vet Med B Infect Dis Vet Public Health* 2000;47:487-95.
69. Corpa JM, Garrido J, García Marín JF, Pérez V. Classification of lesions observed in natural cases of paratuberculosis in goats. *J Comp Pathol* 2000;122:255-65.
70. Gwozdz JM, Thompson KG, Manktelow BW. Lymphocytic neuritis of the ileum in sheep with naturally acquired and experimental paratuberculosis. *J Comp Pathol* 2001;124:317-20.
71. Koh SH, Dobson KY, Tomasovic A. A Johne's disease survey and comparison of diagnostic tests. *Aust Vet J* 1988;65:160-161.
72. Whitlock RH, Wells SJ, Sweeney RW, Van Tiem J. ELISA and fecal culture for paratuberculosis (Johne's disease): sensitivity and specificity for each method. *Vet Microbiol* 2000;77:387-398.
73. Whittington RJ, Fell S, Walker D, McAllister S, Marsh I, Sergeant E, Taragel CA, Marshall DJ, Links IJ. Use of pooled fecal culture for sensitive and economic detection of *Mycobacterium avium* subsp. *paratuberculosis* infection in flocks of sheep. *J Clin Microbiol* 2000;38:2550-6.
74. Eamens GJ, Walker DM, Porter NS, Fell SA. Pooled faecal culture for the detection of *Mycobacterium avium* subsp. *paratuberculosis* in goats. *Aust Vet J* 2007;85:243-51.
75. Eamens GJ, Walker DM, Porter NS, Fell SA. Radiometric pooled faecal culture for the detection of *Mycobacterium avium* subsp. *paratuberculosis* in low-shedder cattle. *Aust Vet J* 2008 ;86:259-65.
76. Kalis CH, Hesselink JW, Barkema HW, Collins MT. Culture of strategically pooled bovine fecal samples as a method to screen herds for paratuberculosis. *J Vet Diagn Invest* 2000;12:547-551.
77. Kalis CH, Collins MT, Barkema HW, Hesselink JW. Certification of herds as free of *Mycobacterium paratuberculosis* infection: actual pooled faecal results versus certification model predictions. *Prev Vet Med* 2004;65:189-204.
78. Wells SJ, Whitlock RH, Lindeman CJ, Fyock T. Evaluation of bacteriologic culture of pooled fecal samples for detection of *Mycobacterium paratuberculosis*. *Am J Vet Res* 2002;63:1207-1211.
79. Wells SJ, Godden SM, Lindeman CJ, Collins JE. Evaluation of bacteriologic culture of individual and pooled fecal samples for detection of *Mycobacterium paratuberculosis* in dairy cattle herds. *J Am Vet Med Assoc* 2003;223:1022-1025.
80. Tavornpanich S, Gardner IA, Anderson RJ, Shin S, Whitlock RH, Fyock T, Adaska JM, Walker RL, Hietala SK. Evaluation of microbial culture of pooled fecal samples for detection of *Mycobacterium avium* subsp. *paratuberculosis* in large dairy herds. *Am J Vet Res* 2004;65:1061-70.
81. van Schaik G, Pradenas F M, Mella N A, Kruze V J. Diagnostic validity and costs of pooled fecal samples and individual blood or fecal samples to determine the cow-

- and herd-status for *Mycobacterium avium* subsp. *paratuberculosis*. *Prev Vet Med* 2007;82:159-65.
82. Raizman EA, Wells SJ, Godden SM, Bey RF, Oakes MJ, Bentley DC, Olsen KE. The distribution of *Mycobacterium avium* ssp. *paratuberculosis* in the environment surrounding Minnesota dairy farms. *J Dairy Sci* 2004;87:2959-66.
  83. Berghaus RD, Farver TB, Anderson RJ, Jaravata CC, Gardner IA. Environmental sampling for detection of *Mycobacterium avium* ssp. *paratuberculosis* on large California dairies. *J Dairy Sci* 2006;89:963-70.
  84. Lombard JE, Wagner BA, Smith RL, McCluskey BJ, Harris BN, Payeur JB, Garry FB, Salman MD. Evaluation of environmental sampling and culture to determine *Mycobacterium avium* subspecies *paratuberculosis* distribution and herd infection status on US dairy operations. *J Dairy Sci* 2006;89:4163-71.
  85. Gwozdz JM. Application of current diagnostic technologies to bulk milk and farm slurry for the diagnosis of Johne's disease in dairy cattle herds. Final Report for Dairy Australia. October 2005 (DAV 11008).
  86. Gwozdz JM, Mohammad I. Improved tools for detection and management of *Mycobacterium paratuberculosis* in animals and food. Final Report for Dairy Australia. November 2008 (DAV 12145).
  87. Tavorpanich S, Muñoz-Zanzi CA, Wells SJ, Raizman EA, Carpenter TE, Johnson WO, Gardner IA. Simulation model for evaluation of testing strategies for detection of paratuberculosis in midwestern US dairy herds. *Prev Vet Med* 2008;83:65-82.
  88. Reddacliff LA, Vadali A, Whittington RJ. The effect of decontamination protocols on the numbers of sheep strain *Mycobacterium avium* subsp. *paratuberculosis* isolated from tissues and faeces. *Vet Microbiol* 2003;95:271-82.
  89. Cousins D V, Evans R J, Francis B R. Use of BACTEC radiometric culture medium and polymerase chain reaction for the rapid screening of ruminant faeces and intestinal tissues for *Mycobacterium. a. paratuberculosis*. *Aust Vet J* 1995;72:458-462.
  90. Eamens GJ, Whittington RJ, Marsh IB, Turner MJ, Saunders V, Kemsley PD, Rayward D. Comparative sensitivity of various faecal culture methods and ELISA in dairy cattle herds with endemic Johne's disease. *Vet Microbiol* 2000;77:357-67.
  91. Whittington RJ, Marsh I, McAllister S, Turner MJ, Marshall DJ, Fraser CA. Evaluation of modified BACTEC 12B radiometric medium and solid media for culture of *Mycobacterium avium* subsp. *paratuberculosis* from sheep. *J Clin Microbiol* 1999;37:1077-1083.
  92. Whittington RJ. Factors affecting isolation and identification of *Mycobacterium avium* subsp. *paratuberculosis* from fecal and tissue samples in a liquid culture system. *J Clin Microbiol* 2009;47:614-22.
  93. Grant IR, Kirk RB, Hitchings E, Rowe MT. Comparative evaluation of the MGIT and BACTEC culture systems for the recovery of *Mycobacterium avium* subsp. *paratuberculosis* from milk. *J Appl Microbiol* 2003;95:196-201.

94. Gumber S, Whittington RJ. Comparison of BACTEC 460 and MGIT 960 systems for the culture of *Mycobacterium avium* subsp. *paratuberculosis* S strain and observations on the effect of inclusion of ampicillin in culture media to reduce contamination. *Vet Microbiol* 2007;119:42-52.
95. de Juan L, Alvarez J, Romero B, Bezos J, Castellanos E, Aranaz A, Mateos A, Domínguez L. Comparison of four different culture media for isolation and growth of type II and type I/III *Mycobacterium avium* subsp. *paratuberculosis* strains isolated from cattle and goats. *Appl Environ Microbiol* 2006;72:5927-32.
96. Whitlock, R. H., A. E. Rosenberger, R. W. Sweeney, and L. J. Hutchinson. Culture techniques and media constituents for the isolation of *M. paratuberculosis* from bovine fecal samples, p. 94–111. In *Proceedings of the 3rd International Colloquium on Paratuberculosis*. International Association for Paratuberculosis. September 1991.
97. Merkal RS. Paratuberculosis: advances in cultural, serologic, and vaccination methods. *J Am Vet Med Assoc* 1984;184:939–943.
98. Merkal RS, Curran BJ. Growth and metabolic characteristics of *Mycobacterium paratuberculosis*. *J Appl Microbiol* 1974;28:276–279.
99. Mathews P, McDiarmid A, Collins P, Brown A. The dependence of some strains of *Mycobacterium avium* on mycobactin for initial and subsequent growth. *J Med Microbiol* 1977;11:53-57.
100. Barclay R, Ratledge C. Iron-binding compounds of *Mycobacterium avium*, *M. intracellulare*, *M. scrofulaceum*, and mycobactin-dependent *M. paratuberculosis* and *M. avium*. *J Bacteriol* 1983;153:1138-46.
101. Thorel MF, Desmettre P. Comparative study of mycobactin-dependent strains of mycobacteria isolated from the wood-pigeon with *Mycobacterium avium* and *M. paratuberculosis*: study of biological and antigenic characteristics. *Ann Microbiol (Paris)* 1982;133:291-302.
102. Gunnarsson E, Fodstad FH. Cultural and biochemical characteristics of *Mycobacterium paratuberculosis* isolated from goats in Norway. *Acta Vet Scand* 1979;20:122-34.
103. Brooks BW, Robertson RH, Corner AH, Samagh BS, Garcia MM, Turcotte C, Duncan JR. Evaluation of the serological response of sheep in one flock to *Mycobacterium paratuberculosis* by crossed immunoelectrophoresis. *Can J Vet Res* 1988;52:199-204.
104. Shulaw WP, Bech-Nielsen S, Rings DM, Getzy MS, Woodruff TS. Serodiagnosis of paratuberculosis in sheep by use of agar gel immunodiffusion. *Am J Vet Res* 1993;54:13-19.
105. Collins DM, Hilbink F, West DM, Hosie BD, Cooke MM, de Lisle GW. Investigation of *Mycobacterium paratuberculosis* in sheep by faecal culture, DNA characterisation and the polymerase chain reaction. *Vet Rec* 1993;133:599-600.

106. Morrison, NE. Circumvention of the mycobactin requirement of *Mycobacterium paratuberculosis*. *J Bacteriol* 1965;89:762-767.
107. Green EP, Tizard ML, Moss MT, Thompson J, Winterbourne DJ, McFadden JJ, Hermon-Taylor J. Sequence and characteristics of IS900, an insertion element identified in a human Crohn's disease isolate of *Mycobacterium paratuberculosis*. *Nucleic Acids Res* 1989;17:9063-73.
108. Englund S, Bölske G, Johansson KE. An IS900-like sequence found in a *Mycobacterium* sp. other than *Mycobacterium avium* subsp. *paratuberculosis*. *FEMS Microbiol Lett* 2002;209:267-71.
109. Cousins DV, Whittington R, Marsh I, Masters A, Evans RJ, Kluver P. Mycobacteria distinct from *Mycobacterium avium* subsp. *paratuberculosis* isolated from the faeces of ruminants possess IS900-like sequences detectable by IS900 polymerase chain reaction: Implications for diagnosis. *Mol Cell Probes* 1999;13:431-442.
110. Motiwala AS, Amonsin A, Strother M, Manning EJ, Kapur V, Sreevatsan S. Molecular epidemiology of *Mycobacterium avium* subsp. *paratuberculosis* isolates recovered from wild animal species. *J Clin Microbiol* 2004;42:1703-12.
111. Rajeev S, Zhang Y, Sreevatsan S, Motiwala AS, Byrum B. Evaluation of multiple genomic targets for identification and confirmation of *Mycobacterium avium* subsp. *paratuberculosis* isolates using real-time PCR. *Vet Microbiol* 2005;105:215-21.
112. Herthnek D, Bölske G. New PCR systems to confirm real-time PCR detection of *Mycobacterium avium* subsp. *paratuberculosis*. *BMC Microbiol* 2006;6:87.
113. Stabel JR, Bannantine JP. Development of a nested PCR method targeting a unique multicopy element, ISMap02, for detection of *Mycobacterium avium* subsp. *paratuberculosis* in fecal samples. *J Clin Microbiol* 2005;43:4744-50.
114. Vansnick E, De Rijk P, Vercammen F, Geysen D, Rigouts L, Portaels F. Newly developed primers for the detection of *Mycobacterium avium* subspecies *paratuberculosis*. *Vet Microbiol* 2004;100:197-204.
115. Strommenger B, Stevenson K, Gerlach GF. Isolation and diagnostic potential of ISMav2, a novel insertion sequence-like element from *Mycobacterium avium* subspecies *paratuberculosis*. *FEMS Microbiol Lett* 2001;196:31-7.
116. Ellingson JL, Bolin CA, Stabel JR. Identification of a gene unique to *Mycobacterium avium* subspecies *paratuberculosis* and application to diagnosis of paratuberculosis. *Mol Cell Probes* 1998;12:133-42.
117. Paustian ML, Amonsin A, Kapur V, Bannantine JP. Characterization of novel coding sequences specific to *Mycobacterium avium* subsp. *paratuberculosis*: implications for diagnosis of Johne's Disease. *J Clin Microbiol* 2004;42:2675-81.
118. Poupart P, Coene M, Van Heuverswyn H, Cocito C. Preparation of a specific RNA probe for detection of *Mycobacterium paratuberculosis* and diagnosis of Johne's disease. *J Clin Microbiol* 1993;31:1601-5.

119. Sockett DC, Carr DJ, Collins MT. Evaluation of conventional and radiometric fecal culture and a commercial DNA probe for diagnosis of *Mycobacterium paratuberculosis* infections in cattle. *Can J Vet Res* 1992;56:148-53.
120. van der Giessen JW, Haring RM, Vauclare E, Eger A, Haagsma J, van der Zeijst BA. Evaluation of the abilities of three diagnostic tests based on the polymerase chain reaction to detect *Mycobacterium paratuberculosis* in cattle: application in a control program. *J Clin Microbiol* 1992;30:1216-9.
121. Collins DM, Stephens DM, de Lisle GW. Comparison of polymerase chain reaction tests and faecal culture for detecting *Mycobacterium paratuberculosis* in bovine faeces. *Vet Microbiol* 1993;36:289-99.
122. Fang Y, Wu WH, Pepper JL, Larsen JL, Marras SA, Nelson EA, Epperson WB, Christopher-Hennings J. Comparison of real-time, quantitative PCR with molecular beacons to nested PCR and culture methods for detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine fecal samples. *J Clin Microbiol* 2002;40:287-91.
123. Christopher-Hennings J, Dammen MA, Weeks SR, Epperson WB, Singh SN, Steinlicht GL, Fang Y, Skaare JL, Larsen JL, Payeur JB, Nelson EA. Comparison of two DNA extractions and nested PCR, real-time PCR, a new commercial PCR assay, and bacterial culture for detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine feces. *J Vet Diagn Invest* 2003;15:87-93.
124. Stabel JR, Bosworth TL, Kirkbride TA, Forde RL, Whitlock RH. A simple, rapid, and effective method for the extraction of *Mycobacterium paratuberculosis* DNA from fecal samples for polymerase chain reaction. *J Vet Diagn Invest* 2004;16:22-30.
125. Taddei S, Robbi C, Cesena C, Rossi I, Schiano E, Arrigoni N, Vicenzoni G, Cavirani S. Detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine fecal samples: comparison of three polymerase chain reaction-based diagnostic tests with a conventional culture method. *J Vet Diagn Invest* 2004;16:503-8.
126. Ellingson JL, Koziczkowski JJ, Anderson JL. Comparison of PCR prescreening to two cultivation procedures with PCR confirmation for detection of *Mycobacterium avium* subsp. *paratuberculosis* in U.S. Department of Agriculture fecal check test samples. *J Food Prot* 2004;67:2310-4.
127. Plante Y, Remenda BW, Chelack BJ, Haines DM. Detection of *Mycobacterium paratuberculosis* in formalin-fixed paraffin-embedded tissues by the polymerase chain reaction. *Can J Vet Res* 1996;60:115-20.
128. Whittington RJ, Reddacliff L, Marsh I, Saunders V. Detection of *Mycobacterium avium* subsp. *paratuberculosis* in formalin-fixed paraffin-embedded intestinal tissue by IS900 polymerase chain reaction. *Aust Vet J* 1999;77:392-397.
129. Odumeru J, Gao A, Chen S, Raymond M, Mutharia L. Use of the bead beater for preparation of *Mycobacterium paratuberculosis* template DNA in milk. *Can J Vet Res* 2001;65:201-5.
130. Kim SG, Shin SJ, Jacobson RH, Miller LJ, Harpending PR, Stehman SM, Rossiter CA, Lein DA. Development and application of quantitative polymerase chain reaction assay based on the ABI 7700 system (TaqMan) for detection and

- quantification of *Mycobacterium avium* subsp. *paratuberculosis*. *J Vet Diagn Invest* 2002;14:126-31.
131. Clark DL Jr, Koziczkowski JJ, Radcliff RP, Carlson RA, Ellingson JL. Detection of *Mycobacterium avium* subspecies *paratuberculosis*: comparing fecal culture versus serum enzyme-linked immunosorbent assay and direct fecal polymerase chain reaction. *J Dairy Sci* 2008;91:2620-7.
  132. Wells SJ, Collins MT, Faaberg KS, Wees C, Tavornpanich S, Petrini KR, Collins JE, Cernicchiaro N, Whitlock RH. Evaluation of a rapid fecal PCR test for detection of *Mycobacterium avium* subsp. *paratuberculosis* in dairy cattle. *Clin Vaccine Immunol* 2006;13:1125-30.
  133. Kawaji S, Taylor DL, Mori Y, Whittington RJ. Detection of *Mycobacterium avium* subsp. *paratuberculosis* in ovine faeces by direct quantitative PCR has similar or greater sensitivity compared to radiometric culture. *Vet Microbiol* 2007;125:36-48.
  134. Yokomizo Y, Yugi H, Merkal RS. A method for avoiding false-positive reactions in an enzyme-linked immunosorbent assay (ELISA) for the diagnosis of bovine paratuberculosis. *Nippon Juigaku Zasshi* 1985;47:111-9.
  135. Jubb TF, Sergeant ES, Callinan AP, Galvin J. Estimate of the sensitivity of an ELISA used to detect Johne's disease in Victorian dairy cattle herds. *Aust Vet J* 2004;82:569-73.
  136. Hope AF, Kluver PF, Jones SL, Condron RJ. Sensitivity and specificity of two serological tests for the detection of ovine paratuberculosis. *Aust Vet J* 2000;78:850-856.
  137. Griffin JF, Spittle E, Rodgers CR, Liggett S, Cooper M, Bakker D, Bannantine JP. Immunoglobulin G1 enzyme-linked immunosorbent assay for diagnosis of Johne's disease in red deer (*Cervus elaphus*). *Clin Diagn Lab Immunol* 2005;12:1401-9.
  138. Salgado M, Manning EJ, Collins MT. Performance of a Johne's disease enzyme-linked immunosorbent assay adapted for milk samples from goats. *J Vet Diagn Invest* 2005;17:350-4.
  139. Hendrick SH, Duffield TE, Kelton DE, Leslie KE, Lissemore KD, Archambault M. Evaluation of enzyme-linked immunosorbent assays performed on milk and serum samples for detection of paratuberculosis in lactating dairy cows. *J Am Vet Med Assoc* 2005;226:424-8.
  140. Gwozdz JM, Thompson KG, Murray A, Reichel MP, Manktelow BW, West DM. Comparison of three serological tests and an interferon-gamma assay for the diagnosis of paratuberculosis in experimentally infected sheep. *Aust Vet J* 2000;78:779-83.
  141. Sergeant ES, Marshall DJ, Eamens GJ, Kearns C, Whittington RJ. Evaluation of an absorbed ELISA and an agar-gel immuno-diffusion test for ovine paratuberculosis in sheep in Australia. *Prev Vet Med* 2003;61:235-48.
  142. Ratnamohan T N, Norris M J, Mitchell G, Spencer T L. A reappraisal of the complement fixation test using soluble *Mycobacterium avium* antigen for the



- detection of *Mycobacterium paratuberculosis* infection in cattle. *Aust Vet J* 1986;63:133-134.
143. Wood PR, Billman-Jacobe H, Milner AR, Carrigan M. Serology and cellular assays for the diagnosis of mycobacterial infections in cattle. In: *Proceedings of the Third international Colloquium on paratuberculosis*. International Association for Paratuberculosis, September 1991.
  144. Kalis CH, Collins MT, Hesselink JW, Barkema HW. Specificity of two tests for the early diagnosis of bovine paratuberculosis based on cell-mediated immunity: the Johnin skin test and the gamma interferon assay. *Vet Microbiol* 2003;97:73-86.
  145. Robbe-Austerman S, Stabel JR, Palmer MV. Evaluation of the gamma interferon ELISA in sheep subclinically infected with *Mycobacterium avium* subspecies *paratuberculosis* using a whole-cell sonicate or a johnin purified-protein derivative. *J Vet Diagn Invest* 2006;18:189-94.
  146. McDonald WL, Ridge SE, Hope AF, Condron RJ. Evaluation of diagnostic tests for Johne's disease in young cattle. *Aust Vet J* 1999;77:113-9.
  147. Jungersen G, Huda A, Hansen JJ, Lind P. Interpretation of the gamma interferon test for diagnosis of subclinical paratuberculosis in cattle. *Clin Diagn Lab Immunol* 2002;9:453-60.
  148. Huda A, Jungersen G, Lind P. Longitudinal study of interferon-gamma, serum antibody and milk antibody responses in cattle infected with *Mycobacterium avium* subsp. *paratuberculosis*. *Vet Microbiol* 2004;104:43-53.
  149. Inderlied CB, Kemper CA, Bermudez LE. The *Mycobacterium avium* complex. *Clin Microbiol Rev* 1993;6:266-310.
  150. Larsen AB, Kopecky KE. Studies on the administration of johnin to diagnose Johne's disease. *Am J Vet Res* 1965;26:673-675.
  151. Buergelt CD, de Lisle G, Hall CE, Merkal RS, Duncan JR. In vitro lymphocyte transformation as a herd survey method for bovine paratuberculosis. *Am J Vet Res* 1978;39:591-595.
  152. de Lisle GW, Duncan JR. Bovine paratuberculosis III. An evaluation of a whole blood lymphocyte transformation test. *Can J Comp Med* 1981;45:304-309.
  153. Hintz AM. Comparative lymphocyte stimulation studies on whole blood from vaccinated and nonvaccinated cattle with paratuberculosis. *Am J Vet Res* 1981;42:507-510.
  154. Elsken LA, Nonnecke BJ. In vitro transformation of lymphocytes from blood and milk of cows with subclinical paratuberculosis. *Am J Vet Res* 1986;47:1513-1516.
  155. Aalund O, Hoerlein AB, Adler HC. The migration test on circulating bovine leukocytes and its possible application for the diagnosis of Johne's disease. *Acta Vet Scand* 1970;11:331-334.

156. Bendixen PH. Application of the direct leukocyte-migration agarose test in cattle naturally infected with *Mycobacterium paratuberculosis*. *Am J Vet Res* 1977;38:1161-1162.
157. Vary PH, Anderson PR, Green E, Hermon-Taylor J, McFadden JJ. Use of highly specific DNA probes and the polymerase chain reaction to detect *Mycobacterium paratuberculosis* in Johne's disease. *J Clin Microbiol* 1990;28:933-937.
158. Moss MT, Sanderson JD, Tizard ML, Hermon-Taylor J, el-Zaatari FA, Markesich DC, Graham DY. Polymerase chain reaction detection of *Mycobacterium paratuberculosis* and *Mycobacterium avium* subsp *silvaticum* in long term cultures from Crohn's disease and control tissues. *Gut* 1992;33:1209-13.
159. Saiki RK, The design and optimization of the PCR. In: Erlich HA, editor. *PCR technology Principles and Applications*. Stockton Press, New York, 1989:7-16.
160. McCreedy BJ, Callaway TH, Laboratory design and work flow. In: Persing DH, editor. *Diagnostic molecular biology. Principles and applications*. Mayo Foundation, Rochester, 1993: 149-159.
161. Wilton S, Cousins D. Detection and identification of multiple mycobacterial pathogens by DNA amplification in a single tube. *PCR: Meth Appl* 1992;1:269-273.

### **Part 3 – Reagents and Kits**

#### **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.

### **Culture Media and Reagents**

#### **Decontamination reagents**

##### **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 minutes. Solution will froth, so fill flasks to only 70% capacity. This solution is stable at room temperature for 1 week only. Do not refrigerate.

For other concentrations of HPC (0.95 or 0.75%) increase or reduce the amount of HPC (9.5 or 7.5 g respectively) in the recipe above to obtain a desired concentration.

### **VAN**

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

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

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

## **Media supplements**

### **Mycobactin J**

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

Mycobactin J 2 mg (2)

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 minutes. 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

---

(2) Allied Monitor, 201 Golden Drive, Fayette, Missouri, USA , 65248

halves. Rupture the egg yolk before addition to the 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 hours.

### **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.

### **Media**

#### **BACTEC 12B vials**

These are supplemented for culture 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

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

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

#### **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 minutes; 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 10 mL volumes into sterile tubes to form slopes. Perform a sterility check by incubation at 37°C for 1 week. Perform

a suitability check by inoculation of *M. paratuberculosis* to demonstrate media ability to support growth of the target organism. Store media at 4°C.

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

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

- 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 6.9-7.0 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 minutes. 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.

### **Absorbed ELISA**

Three commercial, absorbed ELISA kits; Parachek™ (PRIONICS), Pourquier Paratuberculosis Screening Test (IDEXX) and ID Screen® (IDVET) are currently approved for use in cattle in Australia. The Parachek™ kit is also approved for use in sheep and goats. In addition, one test is in use in NZ. The method and interpretation of the results and calculations are fully described in the instructions accompanying the commercial kits.

The Australian Reference positive and negative sera for use with the ELISA for cattle are available from Department of Primary Industries, Attwood, Victoria (3).

### **Complement fixation test (CFT)**

Prepared antigen (*M. avium* strain D4) and Australian Reference Positive Serum used to calibrate the test are available from the Department of Primary Industries, Attwood, Victoria.

*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 g of dried cells added to 200 mL distilled water should have a titre of about 1:50 when titrated against positive sera.

### **Agar gel immunodiffusion (AGID) test**

Prepared antigens of strains 316V and Ama can be obtained from EMAI, Menangle, NSW (4) or of strain 316V from AgResearch Wallaceville Animal Research Centre,

---

(3) [Jacek.Gwozdz@dpi.vic.gov.au](mailto:Jacek.Gwozdz@dpi.vic.gov.au), Tel: 61 3 9217 4200

(4) [graeme.eamens@industry.nsw.gov.au](mailto:graeme.eamens@industry.nsw.gov.au), Tel: 61 2 4640 6358

Upper Hutt, NZ (5). Control positive sheep and goat sera are available from EMAI, NSW.

**Gamma interferon assay**

The BOVIGAM kit is distributed in Australia and New Zealand by AsureQuality, New Zealand (Tel: 64 9 573 8000).