Paratuberculosis (Johne's Disease)

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Part 1 – Diagnostic overview

Summary

Paratuberculosis (Johne's disease) is a chronic enteritis of ruminants caused by infection with Mycobacterium avium *subsp* paratuberculosis (Map)*, a slow growing, Gram-positive, acid-fast bacillus. There are three major strains of this organism; S type (sheep type, Type I), C type (cattle type, Type II) and intermediate type (Type III), which have shown strong host preference in Australia. A further type, known as bison type (B type) can be identified among Type II strains.*

Young animals are most susceptible to infection. However, most clinical cases occur in adults between 2 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 Map *organisms per day in faeces. It is assumed that most animals become infected by ingesting the bacilli.*

Johne's disease is endemic and widespread in sheep, cattle, deer and dairy goats in New Zealand. In Australia, the disease is mainly restricted to parts of temperate south-eastern Australia in dairy and beef cattle, sheep, goats, alpaca, llama and deer. Sporadic cases have been identified in the Northern Territory and Queensland. In the affected areas of Australia, cattle, goats and deer are primarily infected by C strains of Map*, whereas sheep are infected by S strains of* Map*. Johne's disease due to infection with an S strain of* Map *has been detected*

in sheep and goats in Western Australia since 2011. Infection with confirmed C and B strains have been detected in cattle in Queensland since 2012/2013.

The detection of infection depends on the demonstration of Map *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 Map *in the faeces by microscopy, culture, or by the use of DNA-based techniques.*

Diagnosis is made at necropsy by the finding of pathognomonic lesions in the intestines and histological detection of acid fast organisms, or by detection of Map *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. They consist of thickening of the intestinal mucosa, thickening and cording of mesenteric lymphatics and enlargement and oedema of mesenteric lymph nodes, as representations of granulomatous enteritis, lymphangitis, and lymphadenitis, respectively.

Cultures of Map *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* Map *from faeces or tissue is the definitive test for Johne's disease. It is* a *relatively sensitive diagnostic tool and is considered to be 100% specific. Recently, direct polymerase chain reaction testing for* Map *has been shown to be sensitive for herd and flock detection of* Map *infection.*

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 Map.

Aetiology

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

Map is a small (0.5 to 1.5 μm), slow growing, Gram-positive, acid-fast bacillus.[1] This organism shares considerable DNA homology with the organisms previously known as *M. avium* and *M. silvaticum*.[15, 16] 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 taxonomic study based on phenotypic tests.[17] Under defined *in-vitro* growth conditions, *Map* requires supplementation of media with iron-chelating mycobactin for growth.[18]

Several typing methods have been used to investigate the genetic diversity of *Map*.[19] Based on the patterns obtained by IS*900* restriction fragment length polymorphism (RFLP) and pulsed field gel electrophoresis (PFGE) and on growth characteristics and pigmentation, *Map* strains have been divided into three main groups: sheep (S) or Type I strains, cattle (C) or Type II strains and intermediate or Type III strains.[20, 21] Type I and Type III can also be considered as subtypes of the S strain lineage.[22] A single nucleotide polymorphism in the IS*1311* sequence assayed by restriction enzyme analysis (REA) of products of the IS*1311* polymerase chain reaction (PCR) distinguishes S and C strains, consistent with the S (Type I, sheep) and C (Type II, cattle) patterns of the RFLP and PFGE. Although the IS*1311* PCR-REA does not distinguish between Types I and III, it distinguishes C and 'B' (bison) strains within Type II.[23, 24]

There is some host preference evident in Australia from sheep-beef cattle co-grazing observations but cross-species infections do occur. Several studies have demonstrated that regardless of global geographic location, the overwhelming majority of isolates from cattle have been of the C (Type II, cattle) strain. In comparison, most isolates from sheep have been found to be S (Type I, sheep) or I (Type III, intermediate) strains. Most isolates from goats and deer have belonged to C.[19-21, 23-25] A similar, distinct distribution of strains within host populations has been observed in New Zealand and Australia.[20, 26-29] Results of a 2008 study in Australia suggest that based on the current distribution of Johne's disease, the likelihood of transmission of the S strain from sheep to cattle is low.[30] The S strain has also been found to be less virulent for deer than the C strain after experimental exposure.[31] However, emerging evidence from the Cattle Council of Australia's Financial and Non-Financial Assistance (FNF) program in Australian beef cattle, and from New Zealand suggests that 'S' strain is becoming increasingly common where cattle co-graze with infected sheep.

Other genotyping methods suited to epidemiological studies to investigate the movement and origin of *Map* strains in animal populations include variable number tandem repeat (VNTR) procedures, particularly in applications to short sequence repeats (SSR) or mycobacterial interspersed repetitive units (MIRU) of *Map*.[32-34] The tandem application of MIRU and multilocus SSR procedures was recently applied to study *Map* isolates from bovine Johne's disease incursions in QLD.[35] Two strains were identified as the source of the incursions, with no genotypic link to isolates from regions of Australia with a higher prevalence of Johne's disease.

Clinical Signs

The incubation period of Johne's disease is not well defined. Most clinical cases occur in animals between 2 and 5 years of age but it can be delayed for as long as 14 years.[1, 36-38] In a Victorian dairy herd in the 1990's, stressed young bulls (less than 2 years of age) have shown clinical signs of disease (L. Gavey, personal communication 2014). In all ruminant species, the disease is characterised by gradual weight loss and progressive weakness that advances to lethargy and emaciation.[4, 7, 9-11, 36, 37, 39-41]

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.[41] In sheep flocks, the first indication of the disease may be the development of a distinct 'tail' in the mob. This 'tail' is demonstrated by the weaker animals that drop toward the back of the mob when the animals are mustered. Unlike cattle, 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.[4, 9, 36, 37, 39] Similar observations have been reported in llamas and alpacas.[10, 11] Wool break has been reported in sheep[42] and deer may show patchy alopecia,[9] 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.[43] Affected animals may have a short illness with weight loss and diarrhoea. Clinical disease is also observed in older deer.[43]

Epidemiology

Young animals are most susceptible to infection.[44] It has been assumed that most animals which become infected, do so shortly after birth,[1] but recent studies have indicated that infection rates of young and adult sheep are similar.[45] Despite this, sheep exposed as lambs are significantly more likely to shed *Map*, develop lesions and die compared to sheep exposed for the first time as adults.[45] Ingestion of *Map* is believed to be the primary route of infection.

Clinically affected 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 or from pasture.[1, 38] An oral dose of 1,000 organisms (colony forming units) has caused intestinal infection in lambs and young deer[31, 46, 47] but higher doses may be required (from sheep studies).[47] The survival of *Map* in the environment is favoured by low temperature variations associated with protection from direct solar radiation.[48]

The bacteraemia that develops during the course of infection may lead to dissemination of *Map* to other organs (including the udder, sex organs, skeletal muscle)[5, 49-56] and to vertical transmission of infection.[57]

Intrauterine infection of the foetus has been reported in goats, cattle, sheep and deer.[5, 58-61] The percentage of congenitally infected foetuses from cows with clinical Johne's disease ranged from 26.4% to 63.9%,[62] whereas the infection was detected in only 8.6% of foetuses from subclinically infected cows.[59] A recent meta-analysis suggests a significant role for bovine intrauterine infection in Johne's disease epidemiology.[57] In addition, both milk and colostrum may be significant sources of infection, despite the low concentration of *Map* organisms likely to be present in milk.[63] The organism has been isolated from colostrum of 22% of asymptomatically infected cows,[64] from milk of 35% of cows with clinical Johne's disease[65] and from mammary secretions or mammary glands of 3% of clinically affected sheep.[66] Although in cattle, *Map* excretion via milk appears to be correlated with the stage of

infection,[63] 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.[67]

Map has been recovered from bovine and ovine semen,[68, 69] but it is unlikely that venereal transmission plays a significant, if any, role in the epidemiology of Johne's disease.[70]

Map has also been isolated from faeces and tissues of wild ruminants and non-ruminant species.[71-74] Interspecies transmission between wildlife and domestic ruminants occurs, and wildlife may play a role as a reservoir of infection.[74]

Occurrence and distribution

Historically, Johne's disease has occurred in temperate south-eastern Australia in dairy and beef cattle, sheep, goats, deer, and rarely in alpaca and llama. In recent years, infected livestock in Western Australia (sheep, goats) and in Queensland and the Northern Territory (cattle) have been detected. 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 New South Wales in 1980. Further investigations found infected sheep flocks in Victoria, Flinders Island, Kangaroo Island, south-eastern South Australia and mainland Tasmania. The proportion 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 S strain of *Map* was detected in Western Australia followed by the diagnosis of Johne's disease in 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. In 2010 approximately 1,150 cattle herds were 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 identified *Map*-infected sheep and a goat but remains a zone free of bovine Johne's disease in 2014. Western Australia does have periodic introductions of infected cattle, and maintains free zone status normally by eradication in herds where identified cases have occurred. Endemic infection is not known to occur in Queensland or the Northern Territory, but infected cattle properties were detected in Queensland in 2011, 2012 and 2013. Bovine Johne's disease in the Northern Territory has been associated with importation of infected animals from interstate on three occasions in 1976, 1994 and 2001 and was successfully eradicated from each herd with no further spread within or between herds.[75] 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: (http://www.animalhealthaustralia.com.au/programs/jd/jd_home.cfm)

Johne's disease is endemic and widespread in sheep, cattle, deer and dairy goats in New Zealand. The initial diagnosis of Johne's disease in New Zealand occurred in an imported cow in 1912, while ovine Johne's disease was first reported in south Canterbury in 1952 and the first case of Johne's disease in farmed deer was recognized in the late 1980s.[76] 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 generally similar. 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 lymphatics and lymph nodes.[4, 8, 9, 37, 39, 40, 77-80]

The earliest lesions are thickening and cording of mesenteric lymphatics. The mesenteric and ileocaecal lymph nodes may be enlarged and oedematous, and may have focal or diffuse pallor in the cortex. In sheep and deer the lymphatics may have small, white miliary nodules of caseous necrosis along their length.[9,37]

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.[8,37,78-81] Necrotic foci in the intestinal mucosa may be found in goats, sheep and deer.[14] In addition, mucosal hyperaemia, erosions and petechiation have been observed in deer.[4,8] In some goats, sheep and deer, areas of caseous necrosis and mineralisation may be present in the lymph node cortex and appear as white foci 1-4 mm in diameter.[62,78-81] Necrosis, caseation or calcification rarely occur in cattle.[14]

Tubercle-like lesions have also been observed in lymph nodes of the head in deer.[82] Gross changes in deer are very difficult to distinguish from lesions caused by *M. bovis* or other members of the *M. avium* complex.[82, 83]

Diagnostic Tests

The sensitivity of diagnostic tests for detection of *Map* infection in individual animals is relatively low, and assays including histopathology, culture, DNA probes and serological tests have their lowest sensitivity in animals in the early stages of infection. Test sensitivities improve as the mycobacterial load increases, and are generally high in advanced stages of infection.

The accuracy of diagnostic tests is influenced by host factors and the level of exposure to *Map* 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 limitations of test precision mandate that good quality control in specimen collection, handling, storage and laboratory testing is needed to maintain precision at a high level. Positive, negative and inconclusive results occur with all types of tests for *Map* infection.

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 arguably 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, while sensitivity in liquid media has been found to be superior to solid culture (refer 'Part 2: Test Methods - Culture'). 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.[84] The isolation of *Map* from faeces or tissue is considered to be 100% specific.

In sheep, goats, cattle and alpaca, faecal specimens from several individual animals can be combined and culture undertaken on the pooled sample.[85-87] This approach, known as pooled faecal culture (PFC), is used as a screening test. The reported herd sensitivity of the PFC in cattle ranges from 22% to almost 100%.[88-93] In Australia, recommended pool sizes are 5 for cattle, 25 for goats and 50 for sheep. In sheep, herd sensitivity depends on the sampling regimen. In Australian control programs for ovine Johne's disease, PFC is estimated to have a flock sensitivity of 95% in flocks with a prevalence of 2% or more with the recommended regimen of 7 pools of 50 sheep per pool.[94] The specificity of the PFC is considered almost perfect, but it is not uncommon that individual faecal samples from pools tested positive by PFC yield no growth of *Map,* presumably due to low bacterial load and uneven distribution of the organism in the samples.[88-93]

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.[95-97] Australian studies in 2005-2008 indicated that replicate sampling (6 samples/yard) is superior to single sampling for detection of infected herds (J. Gwozdz, personal communication 2010). United States of America (USA) studies using 6 samples/herd and culture on four tubes of solid Herrold's media, have suggested a sensitivity of only 40%[98] whereas in high-shedder herds, sensitivities of 50-78% can be found.[95-97] Sampling of lagoons may be more sensitive than alleyways and cow pens.[96] Commercial liquid culture systems have also been applied to bovine environmental samples in USA prevalence studies and were considered cost-effective.[99]

Among simulated testing strategies, the culture of environmental samples of manure was the most cost-effective for detection of Johne's disease in dairy herds, followed by PFC, individual faecal culture and serum enzyme linked immunosorbent assay (ELISA) with follow-up faecal culture.[100]

Histological examination of tissues has been reported to be less sensitive than culture of tissues in sheep, goats and cattle.[79,101,102] The reasons for this include the small amount of tissue examined microscopically compared to culture, and the examination of different parts of the same organ in the two tests.[38] The specificity of histopathology as a follow-up to a positive ELISA test, where typical lesions are seen (in the intestine and draining lymph nodes) and typical acid-fast organisms are detected, is considered to be 100% in cattle, sheep and goats. While such lesions are required to confirm the presence of Johne's disease in these species, paucibacillary forms of Johne's disease are recognised, particularly in sheep. However, in other species such as deer, lesions may be confused with other infections.

The ELISA is, at present, the most sensitive and specific test for serum antibodies to *Map* in cattle. It detects about 30–40% of cows identified as infected by culture of faeces on solid media.[84] As both culture of faeces and ELISA detect advanced cases most readily, the true sensitivity of ELISA is much lower, with estimates of <16% determined in dairy cattle in Victoria.[103] 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 and is mainly requested for export testing. The agar gel immunodiffusion (AGID) test is sometimes used for testing sheep and goats but not cattle, and may require Chief Veterinary Officer approval

for use in certain applications, such as the ovine Johne's disease market assurance program (OJD MAP).

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 *Map* in clinical specimens such as milk or faeces have been reported, the low sensitivity and specificity of these tests has limited their adoption.[104-111] 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 *Map* in tissues submitted for histopathological examination.[112, 113]

Recent advances in PCR technology and DNA extraction techniques offer new avenues for the development of tests for rapid, sensitive and specific diagnosis of Johne's disease.[114, 115] Results of recent studies are promising,[116-118] with new, improved, direct faecal PCR tests having sensitivities relative to culture ranging from 23%[117] to 70%[116] and specificities ranging from 85.3%[116] to 99.7%.[117] The faecal extraction and PCR test developed by Kawaji et al[118] was 100% specific in 176 *Map*-unexposed, faecal culture-negative sheep and produced positive results in 8/13 (62%) experimentally exposed but faecal and tissue culture negative and histopathology negative sheep; 14/208 (7%) exposed, faecal culture negative sheep; 24/40 (60%) exposed faecal culture negative but tissue culture positive and/or histopathology positive sheep; and in 68/69 (99%) exposed faecal culture positive sheep.

A direct High Throughput Johne's (HT-J) assay for faeces, which incorporates the PCR described by Kawaji et al.[118] has been developed by the University of Sydney and the Elizabeth Macarthur Agricultural Institute (EMAI),[119] and was approved by the Subcommittee on Animal Health Laboratory Standards (SCAHLS) for use in cattle and sheep on a herd/flock basis in 2013. It has been shown to have the same high analytical specificity as the Kawaji et al assay based on a panel of 49 *Mycobacterium* spp other than *Map* including 10 isolates with IS*900*-like sequences[119] reported in two earlier studies.[120, 121] The HT-J assay has been shown to have similar sensitivity to radiometric faecal culture.[119] For example, among 870 cattle in *Map*-infected herds, 67 of the cattle were positive on both HT-J and faecal culture testing, while similar numbers were HT-J positive/faecal culture negative (n=57) compared to faecal culture positive/HT-J negative (n=44).[119] Based on the protocol and criteria for positive results described for the HT-J assay in Part 2 of this document, 62% and 86% of culture positive faecal samples from cattle and sheep respectively were positive in the HT-J assay, whereas the diagnostic specificity relative to faecal culture for individual animals ranged between 98.7 and 98.9% in cattle and sheep.[119]

Safety and Containment Requirements

Safety and containment requirements used in laboratories for common bacterial pathogens apply to work procedures with *Map*. *Map* belongs to pathogen risk group 2, according to the Australian standard for safety in microbiological laboratories (*AS/NZS 2243.3: 2010 Safety in laboratories - Microbiological safety and containment*), requiring PC2 containment.

Part 2 – Test Methods

Histopathology

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.[4, 8, 9, 37, 39, 40, 77-81, 122] In the intestines, these aggregations of macrophages are accompanied by focal or diffuse infiltration of lymphocytes with occasional eosinophils and neutrophils.[37, 39, 40, 78, 80] Multinucleate giant cells may be seen in the intestinal mucosa and cortex of the mesenteric lymph nodes.[77, 78, 81]

In some cases, there are focal to locally extensive aggregates of macrophages and scanty acid-fast organisms (AFOs), together with a lymphocytic infiltrate, in the lamina propria. This type of granulomatous inflammatory reaction is classified as 'paucibacillary' or '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' or 'lepromatous' reaction.[123] The diffuse infiltration of the intestinal mucosa is associated with fusion and atrophy of villi and a decrease in the number of crypts.[37]

In some studies, focal areas of caseation and calcification have been observed in the bowel and mesenteric lymph nodes of sheep and goats.[39, 40, 77-79, 124, 125] However, other workers have either failed to identify such lesions, or have attributed them to parasitic infestation.[37, 80, 122, 126]

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.[9, 82] 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 *Map* from those caused by *M. bovis* and *M. avium*.[82]

Gross lesions in the liver have rarely been reported in sheep,[80] but microgranulomas may be scattered throughout the hepatic parenchyma.[37, 80, 81] In cattle, aggregates of globule leukocytes have been observed in, or around, myenteric ganglion cells.[81] Lymphocytic neuritis in the gut has been reported in sheep.[127]

In all ruminant species, AFOs are usually present within macrophages and multinucleate giant cells in intestinal sections. Numbers of *Map* bacilli in intestinal sections vary from scant to abundant. Fewer AFOs are present in the mesenteric lymph nodes and they are scanty in liver lesions.[37, 80, 81] Standard grading categories have been reported to describe the severity of histopathological changes of Johne's disease in sheep,[80, 122] cattle[52] and deer.[128, 129]

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.

Reagents and Materials

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 below.

Recommended specimens to collect for culture and histopathology are as follows. Intestinal sections of at least 5 cm each for culture and histopathology are advised.

- 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 (10 cm) piece of proximal colon.

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 *Map* infection', examination of one additional section from each block with lesions is recommended.

A positive control consisting of tissues with mycobacteria should be included in the Ziehl-Neelsen procedure. In tissue sections with the correct degree of destaining, the cytoplasm of erythrocytes will retain a pink tinge rather than be heavily counterstained.

Interpretation of Results

A diagnosis of 'lesions consistent with *Map* infection' is indicated if in any one section, one or more single giant cells and/or one or more accumulations of epithelioid macrophages are observed in the intestinal lamina propria and/or lymph node cortex with the presence of at least one AFO morphologically consistent with *Map*.

A finding 'suggestive of *Map* infection' is indicated if in any one section, Langhans' giant cells and/or accumulations of epithelioid macrophages in the intestinal lamina propria and/or lymph node cortex are observed without the detection of an AFO.

When lesions consistent with *Map* infection are present in cattle, sheep or goats (granulomatous inflammation, AFO visible), these results can be interpreted as positive for Johne's disease and should enable appropriate field control measures to be instituted.

Microscopy for AFO's

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 singly and in clumps morphologically consistent with *Map* is consistent with Johne's disease, this is not a definitive test.

Reagents and Materials

See Part 3.

Test Procedure

There are several variations on this staining procedure found in various publications. In general, a hot carbol fuchsin method (Ziehl-Neelsen stain) for mycobacteria is used, and the following is based on well-established clinical pathology procedures in mycobacteriology.[130, 131] Prepare smears and air dry for 10 minutes. Heat fix at 60–70°C. Flood each slide with Ziehl-Neelsen 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.

A cold staining procedure (Kinyoun acid-fast stain) can also be applied for staining mycobacteria in smears. The Kinyoun staining method is similar to the Ziehl-Neelsen stain, but its primary carbol fuchsin stain has a greater concentration of basic fuchsin and phenol, and does not require heating in order to stain properly. After flooding the entire slide with Kinyoun carbol fuchsin stain, the smear is allowed to stain for 2 minutes, then rinsed with water. The slide is then flooded with decolouriser and decolourised until no more colour drains from the slide (approximately 3 to 5 seconds). The slide is rinsed thoroughly with water and any excess moisture shaken off. The slide is flooded with counterstain and allowed to stain for 30 seconds before it is rinsed thoroughly with water and allowed to air dry before examination under oil immersion.

A positive control (smear containing/spiked with *Map*) 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

Principle of the Test

The isolation of *Map* from faeces or tissue is the definitive test to confirm the presence of *Map*, which is critical in the initial diagnosis of Johne's disease in a herd or flock. There are several culture methods, which vary with respect to media, sample type and sample processing protocols. The cultivation of *Map* is always performed using special media supplemented with mycobactin J. Culture of *Map* is a specialised procedure.

Since *Map* 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. Decontamination of faeces is undertaken based on the double incubation/centrifugation method developed in the USA.[132-134] The procedure employs hexadecylpyridinium chloride (HPC) to reduce contaminants in the initial step, half strength brain heart infusion (BHI) broth to promote germination of contaminant spores on incubation, and an antibiotic mixture of vancomycin, amphotericin B and nalidixic acid (VAN) to destroy contaminant vegetative organisms.[134] However, the decontamination process has a negative effect on *Map*. Routine decontamination protocols decrease the number of *Map* organisms by about 2.7 $\log_{10}$ and 3.1 $\log_{10}$ for faeces and tissues, respectively.[135] HPC is recommended as the decontaminant of choice. Despite these decontamination procedures, in Australian studies 11% of bovine faecal cultures,[136] 7% of ovine faecal cultures[137] and 0.13% of ovine tissue cultures[137] in liquid media were mixed (i.e. contained 'contaminating' organisms), while in the USA up to 60% of faecal cultures from cattle were mixed cultures.[138] Decontamination of tissues can be undertaken using either the method described above for faeces, or a simpler method based on HPC decontamination.

There are two techniques for the isolation of *Map*: (i) using solid media and (ii) using liquid media. The latter method can reduce the time required for obtaining a result and is considered to be the more sensitive technique.[102, 136, 139] The decontamination protocol involving double incubation of faecal samples in HPC and a mixture of antibiotics (VAN) may further improve culture sensitivity.[136] The addition of ampicillin to the media has been reported to reduce the growth of undesired microbes.[137, 140]

Most laboratories in Australia and New Zealand use a liquid media culture method for primary isolation, whereas culture on solid media is mainly used to determine mycobactin dependency of isolates. A number of publications have shown that for primary isolation, liquid culture methods have considerably greater sensitivity than solid media culture, regardless of the strain of *Map*.[92, 102, 104, 136, 138, 141, 142] The lower sensitivity of solid media is only partially disguised by the fact that protocols often specify inoculation of multiple (up to four) solid media slopes.[138] Liquid culture is recommended for primary culture of *Map* in Australia.

Samples

Collection procedures for bacteriological tests should avoid contamination of specimens with environmental fungi or bacteria. Sample jars must be refrigerated after collection. Specimens should be refrigerated for transport to the laboratory (using at least a chiller brick in an insulated box). 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^{o}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. Use separate clean gloves when multiple individual samplings are made.

Faeces for PFC test (cattle, alpaca, sheep and goats)

For the PFC 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. A change of gloves between individuals in a pool is recommended when groups of animals with high value or high risk of infection are sampled, to avoid later misidentification of infected individuals. Pooling of cattle faeces is best done in the laboratory, to ensure that the pooled sample contains an equivalent volume from each animal. 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, to enable later follow-up of individual animals if required.

Tissues

Collect small (approximately 10 g) pieces of relevant sections of small intestine, large intestine and associated lymph nodes (refer above section 'Histopathology' for recommended tissues) into sterile leak proof plastic containers using aseptic technique.

Reagents and Materials

There are several liquid media culture systems available for *Map* but only one has been validated in Australia (M7H9C, University of Sydney).[143] It is a modified non-radiometric Middlebrook 7H9 broth media with additives and replaces the radiometric BACTEC 460 culture system which was based on BACTEC 12B media (Becton Dickinson). Like the former system, M7H9C is suitable for cultivation of S and C strains of *Map*. The incubation time is 12 weeks for C and S strains, and requires PCR testing of all cultures. Manufacture of the M7H9C medium is based on commercial Middlebrook 7H9 base to which casitone (667 mg/L) is added, and the media is supplemented with commercially-available ADC enrichment (albumin faction V, bovine; dextrose; catalase) (26.7 mL/L), egg yolk (167 mL/L), mycobactin J (0.83 mg in 16.7 mL/L) and an antibiotic mixture (polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin). Among other liquid media culture systems, MGIT 960 (Becton Dickinson) is only suitable for cultivation of C strains.[140, 144]

There are two commonly used solid media for the diagnostic isolation of *Map* in Australia and New Zealand: Herrold's egg yolk agar (HEY) and modified Middlebrook 7H10.[102] Both media are supplemented with egg yolk and mycobactin. The latter media supports the growth of both S and C strains,[102] whereas HEY primarily supports the growth of C strains.[145, 146] Although there are reports that Lowenstein-Jensen (LJ) media with mycobactin is suitable for the isolation of S and C strains found in Europe, in general very prolonged incubation periods are needed for these media.[145, 146] 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.[134, 145, 147, 148] Other media, such as Dubos media and Watson-Reid media, are not recommended for routine culture of clinical specimens.

Primary colonies of *Map* on solid media may be expected to appear any time after 3 weeks following inoculation, but may not appear for months. 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, as some may take 6-8 months,[145] although incubation beyond 20 weeks is less practical.

Colonies of the cattle strain of *Map* on HEY media 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 media, colonies of the cattle strain are less convex than those on HEY, especially in aged cultures; are pinpoint to approximately 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 media) and to differentiate from colonies of some other mycobacteria.

Colonies of the sheep strain of *Map* on modified 7H10 are convex, shiny, raised, white to off-white, and difficult to distinguish against the background colour of the media. Colonies are typically between pinpoint and 0.5 mm in diameter, but can reach 1 mm, and rarely 1.5 mm if few colonies occur on a slope.[102, 146]

Colonies of *Map* often appear as a mixed culture with other taxa. Saprophytic mycobacteria may have a similar appearance on either media but are often evident after 5-7 days. Other organisms may grow on both media with colonies appearing after days or months.

Test Procedures

A range of volumes of sample materials and their associated solutions have been successfully applied for *Map* culture, with a general aim to provide sufficient source material but reduce levels of potential contaminants. The protocols described here afford a range of such volumes.

Decontamination of individual faeces for culture

Method 1: Mix 1.2 to 3 g faeces with 10-15 mL saline or water. After 30 minutes sedimentation, the top 3-5 mL is transferred to 20-25 mL 0.9% HPC in half-strength BHI broth. (It is important to avoid transfer of the faecal sediment, so the volume transferred may be variable). After incubating at 35-37ºC for 16–26 hours, the inoculated HPC in half-strength BHI is centrifuged at 900-1200 ***g*** for 30 minutes (keep temperature >10ºC to avoid precipitation).

Method 2: Alternatively, 2 g of faeces is mixed vigorously in 35 mL 0.75% HPC in half-strength BHI broth and after incubation as above, 20 mL of the supernatant is transferred (avoiding fibrous sediment) and centrifuged as above.

Discard the supernatant fluid and re-suspend the pellet in 1 mL of VAN or VAN/BHI solution. Incubate at 37ºC for 24–72 hours (72 hours is recommended to minimise contamination) and 0.1 mL of the re-suspended pellet is inoculated into the culture media.

Homogenisation of pooled faeces

Work must be conducted in a manner that minimises the risk of sample-to-sample cross-contamination. The method of homogenisation of pooled faeces prior to culture or HT-J faecal PCR is dependent on the sample type and availability of equipment. If it is desired to prepare a common suspension for use in culture and for testing by direct faecal PCR using the HT-J protocol, refer to the 'High Throughput Johne's (HT-J) Direct PCR Assay' section for the

appropriate volume and step for inclusion of pooled material.

Method 1: Homogenisation using a Waring commercial blender. Faecal pellets from up to 50 sheep or 25 goats, or faecal samples (2 g each) from five cows or five alpaca are completely homogenised using the Waring commercial blender base with 250 mL stainless steel blenders, without the addition of saline. For culture and HT-J, the homogenised faecal sample prepared in this way may be treated the same as an individual faecal sample. Refer to the section on 'Decontamination of individual faeces for culture' for sedimentation method.

Method 2: Homogenisation using a stomacher system with added saline. The supernatant from the sedimentation step for each of the variants described below (2.1, 2.2 and 2.3) is suitable to proceed to 'Decontamination of pooled faeces for culture'.

Method 2.1: In sheep or goats, the submitted faecal sample (pooled pellets from up to 50 individual sheep or 25 goats) is weighed, added to a blender/stomacher bag containing a volume of 0.85% saline (1:1) and homogenised for a minimum of 1 minute. For a 1:1 homogenisation mixture, a universal screw-topped container with 50 sheep pellets contains approximately 40-50 g, so requires 40-50 mL saline. From this homogenate, 3-4 mL of the mixture is transferred to a 20-25 mL screw-topped tube (or centrifuge tube) containing 10 mL of saline. After mixing by hand, the material is allowed to settle for 30 minutes (but not more than 60 minutes).

Method 2.2: In cattle and alpaca, faecal samples (2 g each) from five cows or five alpaca are added to a blender/stomacher bag containing a volume of 0.85% saline (1:1) and homogenised for a minimum of 1 minute (total 10 g of pooled faeces in 10 mL saline). From this, 3-4 mL is removed and added to 10 mL saline for sedimentation as described for method 2.1.

Method 2.3: Alternatively, for cattle and alpaca, a larger saline volume can be used; for example a total of 10 g pooled faeces can be mixed with 50 mL saline (1:5), of which 10 mL is removed post homogenisation and added to 10 mL saline for sedimentation as described for method 2.1.

Decontamination of pooled faeces for culture

3-5 mL of supernatant from the sedimentation tube (containing saline) is added to a 25 mL tube containing 20 mL of 0.9% HPC in half-strength BHI, mixed, incubated at 37°C for 16–26 hours and centrifuged at 900-1200 ***g*** for 30 minutes (keep temperature > 10°C to prevent HPC precipitation). After discarding the supernatant, the pellet is re-suspended in 1 mL of VAN or VAN/BHI, incubated at 37°C for 72 hours and 0.1 mL of the re-suspended pellet is inoculated into the culture media.

A general procedure for individual faecal culture is shown in Figure 1.

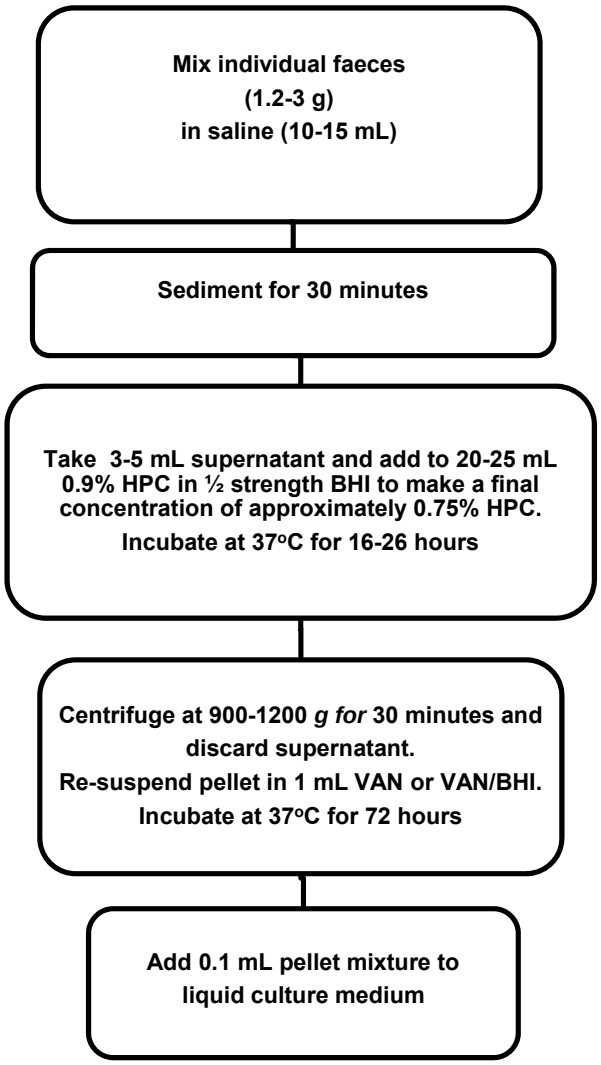

Figure 1. Steps in *Map* culture from an individual faecal sample based on centrifugation/double incubation method 1.

General procedures for pooled faecal culture are shown in Figure 2. Supernatant after the sedimentation step can be used for direct PCR (HT-J) testing; refer to the 'High Throughput Johne's (HT-J) Direct PCR Assay' section for the appropriate volume and step for inclusion of pooled material.

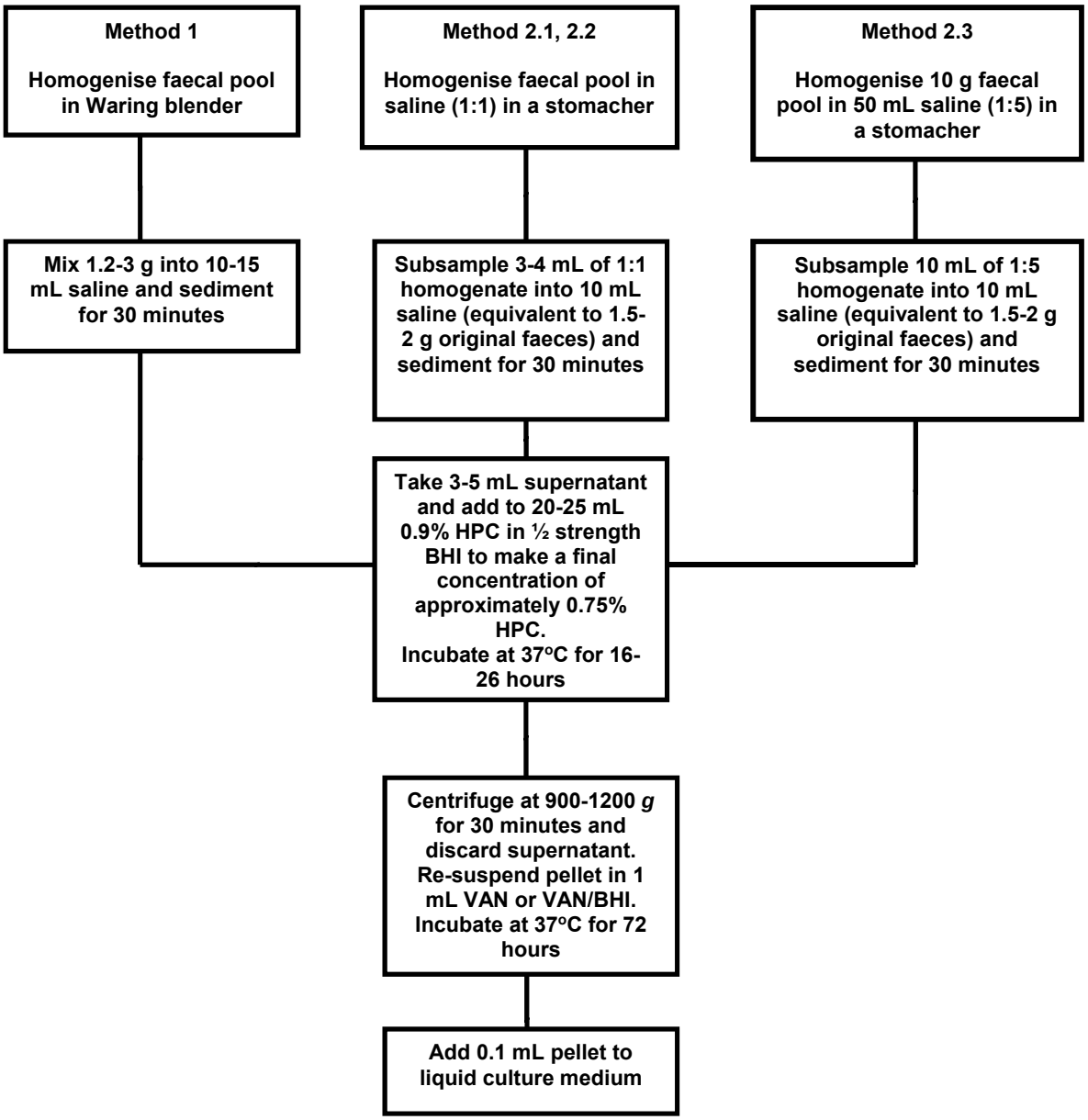

Figure 2. Steps in *Map* culture from a faecal pool based on centrifugation/double incubation method, with homogenisation using either a Waring blender without saline (top left) or stomacher-type systems with saline (top centre and top right).

Decontamination of tissues for culture

Two methods to disrupt and decontaminate tissues have been applied successfully in Australia. One is based on the double incubation/centrifugation method used for faeces while the second uses a sedimentation technique with decontamination of homogenised tissue in 0.75% HPC for 72 hours and inoculation of sediment. In general the culture contamination rate for tissue samples is very low, suggesting that both approaches are highly effective.

Method 1: Finely chop 2 g of tissue sample (trimmed of fat) using a sterile scalpel blade or scissors and homogenise 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 37ºC for 3 hours. Centrifuge at 900 ***g*** for 30 minutes, discard the supernatant fluid and re-suspend the pellet in 1 mL of VAN or VAN/BHI. Incubate at 37ºC overnight. Inoculate media as described below. Alternatively, a sedimentation technique for tissues involves decontamination of homogenised tissue in 0.75% HPC for 24 to 72 hours and inoculation of sediment onto media.

Method 2: With sterile forceps, finely chop 2 - 5 g of tissue sample (trimmed of fat) using a sterile scalpel blade or scissors and add to either a glass homogeniser jar or a 80-100 mL stomacher bag containing 2-4 mL sterile 0.85% w/v saline solution. Homogenise in the homogeniser jar for approximately 10-30 seconds at full speed (increasing speed gradually) or in the stomacher for 2 minutes. Note that if using an homogeniser, do not allow the specimen to heat up through prolonged homogenisation. Avoiding any obvious pieces of tissue, transfer 2 mL of tissue homogenate to 25 mL of 0.75% HPC (or 2-5 mL homogenate to 30 mL of 0.75% HPC) in a sterile 30-35 mL polycarbonate tube. Mix by inverting a couple of times, then incubate at room temperature (approximately 23ºC), away from light for 72 hours. Avoid disturbing the HPC and inoculate 0.1 mL of the sediment layer at the bottom of the tube into 7H9 liquid media.

Inoculation and incubation of solid media cultures

Primary culture onto solid media is not recommended as a routine procedure due to its lower sensitivity compared with liquid culture. Although solid media can be used successfully with samples from animals with high concentrations of *Map* in faeces or tissues (such as clinical cases), solid media is only recommended for routine use as a subculture from liquid 7H9 broth to confirm mycobactin dependence, colonial morphology and to obtain purified material for strain typing or storage/lyophilisation. It is important to note that a false negative culture outcome rate of 30% can be expected when liquid media is subcultured to solid media due to the lower capacity of solid media to support growth.[137] As animals showing clinical signs consistent with Johne's disease may be infected with *Map* at low levels but have clinical signs due to an unrelated condition, laboratory testing of samples using primary solid media for cultural confirmation should seek evidence to confirm high level shedding or infection (e.g. by demonstration of Ziehl-Neelsen positive smears from faeces or tissues) is present.

For cattle or sheep strains, culture of decontaminated sample material or 12 week broth culture material can be undertaken by adding 0.1 mL to 2-3 slopes of modified 7H10 media supplemented with 2 µg/mL mycobactin J. For cattle strains only, culture to HEY supplemented with 2 µg/mL mycobactin J (HEYM) and sodium pyruvate and onto one slope of HEYM without sodium pyruvate can be used. Incubate slopes at 35-37ºC for at least 4

months but preferably 6 months. Subsequent subculture of colonies resembling *Map* onto media with and without mycobactin is used to demonstrate mycobactin dependence. Care should be taken not to transfer excessive amounts of culture or parts of the solid media, as this can inadvertently transfer a small amount of mycobactin and make mycobactin dependency testing invalid.

Inoculation and incubation of M7H9C broth cultures

Inoculate 0.1 mL of decontaminated sample into one supplemented M7H9C tube. Incubate at 35-37ºC for 12 weeks for all samples (i.e. for cattle and sheep strains). After the 12 week incubation assess all cultures by IS*900* PCR tests recommended in the section 'PCR testing to confirm *Map'* below. If desired, subculture to solid media for mycobactin dependence testing can be carried out at this stage.

Negative and positive controls must be included in each batch of specimens for culture. Ideally the positive samples should be authentic faecal samples from animals with Johne's disease, but spiked faeces may be used if authentic samples are not available.

Interpretation of Results

The identification of *Map* isolates commonly relies on the slow growth rate of acid-fast bacilli, demonstration of mycobactin dependency and detection of IS*900,* a DNA fragment that is considered to be unique for the *Map* genome.[15, 16] Ziehl-Neelsen staining of colonies to demonstrate acid-fastness is not obligatory for operators with extensive experience in recognition of typical colonies of *Map*, but is recommended where there is doubt that colonies are typical of *Map*. Similarly, the Ziehl-Neelsen stain may be applied on liquid cultures to confirm the presence of acid fast organisms. However, mycobacteria other than *Map* may be present and cannot be distinguished from *Map*, and the sensitivity of Ziehl-Neelsen staining is low relative to PCR.

Mycobactin Dependency Assay

Principle of the Test

For the demonstration of mycobactin dependency, a small inoculum of suspect colonies should be subcultured on solid media 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 *Map* on the media without mycobactin, leading to misidentifications and false negative results. Mycobactin dependency tests for cattle strains can be performed on HEY media or modified 7H10 media, whereas tests on the sheep strain must be performed using modified 7H10 media.

In addition, the identification process may be confounded by infections caused by mycobactin-dependent *M. avium* subsp. *sylvaticum* and *M. avium* subsp. *avium* strains,[149-151] the difficulty in the isolation of some ovine strains of *Map*,[37, 152-154] variations in mycobactin-dependence of the organism under different culture conditions,[155] and presence of IS*900*-like genes in mycobacteria other than *Map*.[121]

Test Procedure

One colony from the solid media is mixed in 0.5 to 1 mL of phosphate-buffered saline (PBS, pH 7.2). Subculture 0.1 mL volumes of the prepared suspension or liquid culture 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.

Interpretation of Results

Acid-fast bacilli that show significantly enhanced growth on media containing mycobactin after at least 2 weeks incubation, with no growth on an un-supplemented slope, are considered consistent with *Map*. Acid-fast bacilli not dependent on mycobactin are classified as other mycobacteria (not *Map*).

PCR Testing to Confirm *Map*

Principle of the Test

Since the discovery of the first *Map*-specific repetitive insertion segment IS*900* by Green et al. (1989),[156] a number of primers that target IS*900* have been published.[106, 118, 157-165] Their specificity was evaluated using different numbers of *Mycobacterium* spp and other common bacteria.[159, 161, 164-167] The occurrence of IS*900*-like sequences in non-*Map* isolates is extremely rare but has caused some uncertainties about the specificity of PCR systems targeting IS*900* for routine diagnosis. Over several decades of testing hundreds of thousands of samples of suspect *Map* cultures, IS*900*-like sequences have been found in three non-*Map* isolates related to *M. scrofulaceum*[121] and one related to *M. cookie,*[120] and false positive results obtained by IS900 PCR for some *Mycobacterium avium* complex (MAC) strains and non-*Map* isolates including two unidentified mycobactin-independent isolates, *M. terrae, M. xenopi* and *M. chelonei.*[121, 167-170] Cousins *et al* (1999) found IS*900*-like sequences in Australian *M. scrofulaceum*-like organisms were present in fewer copy number than in *Map* and were able to be differentiated from IS*900* in *Map* by restriction endonuclease analysis.[121] As a general guide, primers must be selected from the specific (5') end of IS*900.* In addition, increased annealing temperatures of 62-68^{o}C have been recommended to ensure specificity of conventional IS*900* PCR used in Australia,[162] as well as use of new IS*900* primers that are not cross-reactive with other mycobacteria.[118, 164]

The identification of new DNA fragments considered unique to *Map* has provided additional targets for rapid identification of this organism using PCR technology. Such sequences include ISMap*02*,[171] ISMav*2*,[172, 173] f57,[164, 174-176] hspX,[177] and locus 255.[178, 179] Assays for the new sequences have been developed for standard (gel-based) or quantitative (real time)[118] PCR platforms, with the former including single[172, 173] or nested[164, 171] applications. Multiplex real time PCR assays that target various combinations of these sequences have been reported (e.g. IS*900*, f57 and ISMap02,[180] or f57 and ISMav2[181]). However, the PCR tests based on new sequences usually have reduced analytical sensitivity due to lower copy numbers of target DNA in the *Map* genome compared to IS*900*.[172, 174, 177, 182] While IS*900* is present in 15-20 copies[156] (average 17 copies[171]) in the *Map* genome, f57 and locus 255 are single copy genes,[180] ISMav*2* is present in 3 copies,[180] and ISMap*02* is present in 6 copies.[171] Due to copy number differences it is not uncommon for assays of samples that contain *Map* to yield negative results for f57 whereas IS*900* is positive;[183-185] this is not useful in a diagnostic setting. In addition, conventional PCR assays that use a nested approach require high levels of containment to avoid amplicon contamination, thus posing difficulties for routine diagnostic application, and so are not recommended. Redesign of some published primers for

the newer sequences may also be required if evidence of cross-reactivity is confirmed. Mobius et al[179] reported that stringent selection of IS*900*-specific primers will ensure that IS*900* remains a favourite target sequence for amplification of *Map* specific loci. They found single round standard PCR systems based on f57 and locus 255 were reliable, but revision of ISMav*2* primers was necessary.

For the identification of culture-derived isolates, a PCR test based on primers that target a specific segment of the insertion sequence IS*900* is recommended. Conventional IS*900* PCR tests using primers described by Vary *et al* (IS*900*/150C, IS*900*/921, 229 bp target)[159] or Moss *et al* (P90, P91, 400 bp target)[186] as modified by Millar *et al* ($P90^+$, $P91^+$, 413 bp target)[160] have been used successfully in Australia, while for real-time assays, the primer set described by Kawaji *et al* (MP10-1, MP11-1, 183 bp target)[118] is recommended. The quantitative IS*900* PCR methodology used to detect *Map* as part of the HT-J direct faecal quantitative PCR (qPCR) protocol[119] incorporates the MP10-1 and MP11-1 primers from Kawaji *et al* and is sensitive and specific for use in the detection of *Map* in liquid cultures (K. Plain, personal communication 2014). Primers for the *Mycobacterium* genus based on the 16S rRNA sequence can also be included in the PCR mix to produce a multiplex conventional PCR that can differentiate *Map* from other *Mycobacterium* spp that grow in liquid or on solid media.[187] Restriction endonuclease analysis of conventional IS*900* PCR product with an enzyme such as *Hae* III (in the Vary system) or *Mse* I (in the Moss/Millar system) is recommended to achieve high specificity of the assay.[121]

All PCR methods, both conventional and real-time variants, should be validated and optimised in each laboratory to determine, for example, the optimum concentrations of each of the essential components in the reaction (primers, enzyme, $MgCl_2$ and nucleotides) and the temperature and time of each of the cycles used in amplification.[188] Appropriate laboratory practice should be instituted to prevent contamination.[189]

Special Requirements for PCR-based Methods

Preparation of samples from colonies on solid media

A sample of colony growth (normally one colony is sufficient) is mixed in 100 µL of purified sterile water, or a commercial DNA preparation solution (e.g. Prepman Ultra, ABI). The suspension is heated at 95-100ºC for 10-15 minutes and centrifuged at high speed (14,000 ***g*** in a standard bench-top centrifuge). At this stage, supernatant can be stored frozen at -20ºC. Dilution of the supernatant as template for PCR may be required to obtain a positive result.

Preparation of samples from liquid culture

The presence of egg yolk has been found to inhibit the PCR reaction. At the end of the incubation period this can be overcome by:

(a) subculture into liquid media without egg yolk; or
(b) by removing the egg yolk from the primary culture by alcohol precipitation; or
(c) using a commercial extraction kit known to effectively reduce PCR inhibition in the PCR platform to be utilised; but this approach requires validation.

Procedures (a) and (b) above can be performed as follows:

(a) Inoculate 100 µL of the positive sample into liquid 7H9 media with mycobactin J but without egg yolk or PANTA. Incubate the subculture for about 14 days, at which stage 200 µL is removed and heated to 100ºC for 30 minutes to make ready for testing.[139]

(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, re-suspended in 50 µL sterile purified water and heated at 100ºC for 20 minutes to make ready for testing.[102]

Preparation of samples from fixed tissue

A sample of paraffin-embedded tissue is prepared for PCR according to Whittington *et al* (1999).[113]

Test Procedure

Conventional IS900 PCR procedures

A minimum annealing temperature of 60ºC and 35 cycles of amplification should be used for IS*900* primers. Suggested primers and products for IS*900* PCR application to culture material or fixed tissue are outlined in Table 1, while controls that must be run with each PCR or REA are shown in Table 2.

For the 900M and 900V reaction, the PCR conditions are as described by Whittington et al,[102, 142] with a single denaturation cycle of 94ºC for 2-3 minutes, followed by 37 cycles of denaturation at 94ºC for 30 seconds, annealing at 62ºC for 15 seconds and extension at 72ºC for 1 minute. The 900M primers are as follows: P90: GAA GGG TGT TCG GGG CCG TCG CTT AGG; P91: GGC GTT GAG GTC GAT CGC CCA CGT GAC. The 900V primers are as follows: 150C: CCG CTA ATT GAG AGA TGC GAT TGG; 921: AAT CAA CTC CAG CAG CGC GGC CTC G.

Table 1. Suggested IS*900* PCR reactions

Source material	Target	Reaction (name)	Forward Primer	Reverse Primer	Predicted Product (bp)	Reference
Culture	IS*900*	900 M	P90+	P91+	413	Millar *et al* 1996 [160]
Fixed tissue	IS*900*	900 V	150C	921	229	Whittington *et al* 1999 [113] Vary *et al* 1990 [159]

Table 2. Controls required for IS*900* PCR and REA assays

Control type	PCR controls	REA controls
Positive	Extraction from *Map* positive ovine faeces or tissue	PCR product from extraction positive control
Positive	*Map* cattle strain	PCR product from *Map* cattle strain control
Positive	-	Uncut PCR product from

		Map cattle strain control (i.e. not exposed to enzymes)
Negative	PCR MilliQ water (PCR MQW)	REA MilliQ water (REA MQW)
Negative	PCR cocktail only	REA cocktail only
Negative	Extraction from negative control faeces/tissue	-

For conventional (standard) 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 or 1 × GelRed® (Biotium Inc.) or equivalent alternative to ethidium bromide. Amplified product is visualised using an ultraviolet transilluminator and photographed. The size of the amplified products is estimated after comparison with an appropriate molecular weight ladder and the expected size of the IS*900* product based on the positive control. This will depend on the primer sequences selected for use in the PCR reaction.

Where a multiplex PCR is used for *Map* confirmation as described by Wilton and Cousins 1992,[190] a positive result 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 IS*900* sequence. Other *Mycobacterium* spp should produce only the genus band in the multiplex PCR.

*REA procedures on conventional IS*900 *PCR product*

REA of the amplified product is recommended for routine use of conventional IS*900* PCR to confirm that the sequence of the amplified product is consistent with the known *Map* sequence. Alternatively, the amplified product can be sequenced in its entirety. The isolate may also be tested for other DNA fragments that are considered unique for *Map,*[164, 171, 172, 177] although the sensitivity of such assays will be lower than those based on IS*900*.

For conventional PCR, results of laboratory testing may range from a trace reaction to a strong band of appropriate molecular size in the gel. REA can only be performed on IS*900* amplified products that show a specific gel band (i.e. a positive result). The volume of amplified product required for REA is determined according to the intensity of the observed specific amplified product. As a guide, a volume range for the different PCR reactions is shown in Table 3, where a PCR reaction grade 4+ is a very strong positive gel line, and a 0.5 to trace represents a barely visible line of reaction.

In the case of IS*900* PCR using the Vary primers, the *Hae* III REA digest results in visible fragments of 60 and 137 bp from *Map* (two smaller fragments of 12 and 20 bp are generated but will not be visible), whereas in the Moss/Millar system, the *Mse* I digest produces fragments of 130 and 283 bp. Other enzymes may be used, based on logical choice from DNA sequence data.

Table 3. Volumes suggested for REA depending on strength of relevant PCR reaction

IS*900* PCR reaction grade	Volume range suggested for REA (µL)
4+	1-5
3+	2-6

2+	5-12
1+	10-19.2
0.5 to trace	19.2

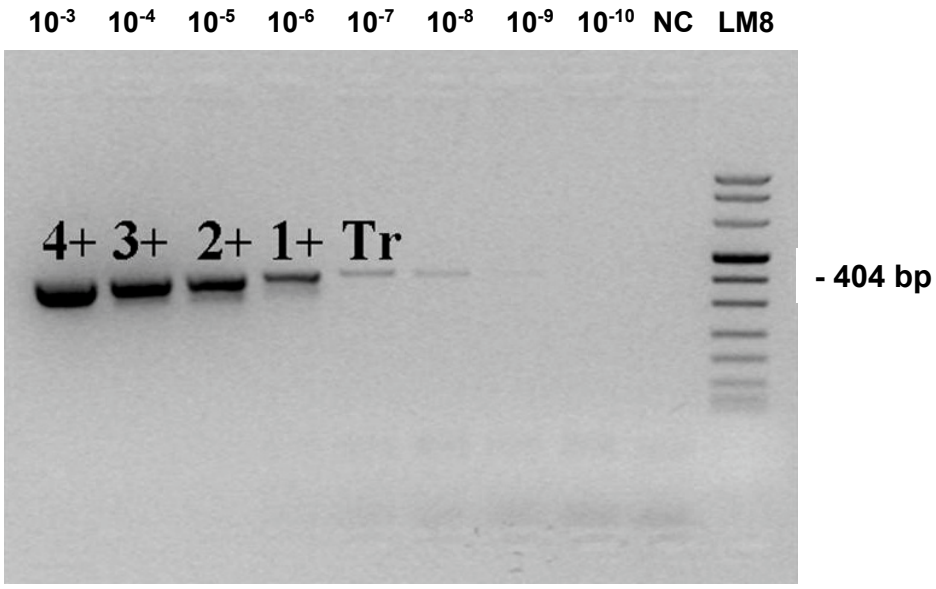

Figure 3. Suggested grading of amplified product for REA estimation. This figure is derived from an IS*900* PCR using the 900M reaction and a 10 fold DNA dilution series. 20 μL of PCR product was added to the gel directly from the PCR tube after adding 4 μL of loading dye. Dilutions are shown at the top of each lane. NC: negative control. LM8: molecular weight ladder, showing 404 bp marker relative to the expected specific 413 bp product.

The REA cocktail volume is determined by the number of samples plus 1 spare for every 10 tubes. The following tables can be used as a guide to calculate the cocktail reagent volumes for IS*900* REA reactions. When gel bands of 2+ to 4+ are found in the 900M, 900V PCR, routine REA volumes of 16 μL according to Table 4 can be applied. For weak gel bands of up to 1+, reactant volumes need to be increased to 25 μL according to Table 5.

Table 4. Reagents for routine REA for 900M or 900V, for a volume of PCR product (X) to a maximum of 12.3 μL

Reagent	Volume per tube (μL)	Final concentration/amount
MQW	Up to final volume	
10 × NE Buffer 2	1.6	1 ×
10 × BSA (1μg/μL)	1.6	1.6 μg

Mse 1 (4 U/μL)	0.5	2 U
Cocktail Volume	16-X	
PCR product	X	
Final Volume	16	

Table 5. Reagents for special REA reaction for 900V or 900M for a volume of PCR product (X) to a maximum of 19.2 μL

Reagent	Volume per tube (μL)	Final concentration/amount
MQW	Up to final volume	
10 × BSA (1μg/μL)	2.5	2.5 μg
Mse 1 (4 U/μL)	0.8	3.2 U
Cocktail Volume	25-X	
PCR product	X	
Final volume	25	

For IS*900* REA, 3% agarose is typically used to visualise the results of electrophoresis, with appropriate molecular weight markers (e.g. Roche Molecular Weight Markers VIII). The entire volume of digested product (including loading buffer) is run. The predicted REA band sizes for IS*900* assays 900M and 900V are shown in Table 6, while Figure 4 shows the expected gel profile for *Map*.

Table 6. Predicted band sizes for *Map* IS*900* REA based on reactions 900M and 900V. Uncut cattle strain control will show a single band at 413 or 229 bp depending on the reaction type.

REA reaction	PCR product (bp)	Enzyme	Species	Predicted REA bands (bp)
900M	413	*Mse* 1	*Map*	130, 283
900V	229	*Mse* 1	*Map*	70, 159
900V	229	*Hae III*	*Map*	12[a], 20[a], 60, 137

[a] Bands at 12 and 20 bp not visible

*IS*900 *PCR and REA quality control acceptance criteria*

For the IS*900* PCR, the negative controls (PCR MQW, PCR cocktail, negative sample control) must show no bands. The positive controls (extraction positive control, cattle strain positive control) will show a single band equivalent to the original target of the PCR assay used.

For the IS*900* REA, the negative controls (REA MQW, REA cocktail) must show no bands. The positive controls (extraction positive control, cattle strain positive control, uncut cattle strain positive control) will show bands as indicated in Table 6.

Samples found positive in the standard IS*900* PCR/REA should be interpreted as containing 'DNA consistent with *Map'*.

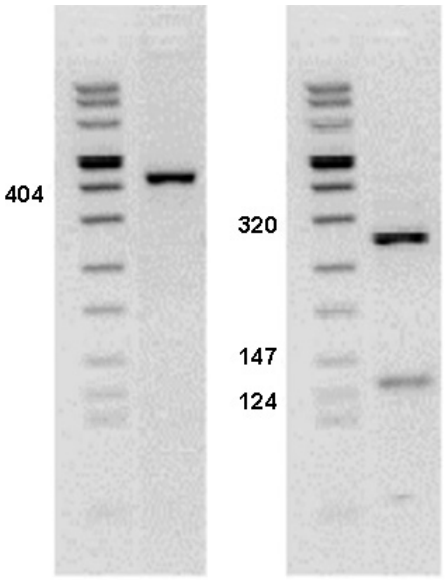

Figure 4. IS*900* profiles of *Map* (900M) before REA (left) and after REA (right).
Lanes 1, 3: Molecular weight markers
Lane 2: Pre-digest with 413 bp band
Lane 4: Post-digest with 130 and 283 bp bands

*IS*900 *real-time PCR procedures*

The primer set described by Kawaji *et al* (MP10-1, MP11-1, 183 bp target)[118] is recommended. The quantitative IS*900* PCR methodology used in the HT-J assay, described in this ANZSDP, incorporates the MP10-1 and MP11-1 primers from Kawaji et al (refer to the 'High Throughput Johne's (HT-J) Direct PCR Assay' section for the method).[119] For real-time PCR systems used to identify *Map*, the amplification curve of each sample and a melt curve analysis (if applicable) need to be visually examined for the presence of exponential and plateau phases and T_m consistent with controls, respectively. Other interpretation criteria related to limit of detection and limit of quantification may be required, depending on purpose and reaction chemistry. In general C_T >40 are not meaningful.[191]

In real time PCR, results should be recorded as a DNA concentration and interpreted relevant to a positive-negative cut-point. Samples found positive in the IS*900* real time PCR should be interpreted as containing 'DNA consistent with *Map*.'

Strain typing of *Map* samples and cultures by PCR

Principle of the Test

Rapid molecular typing of *Map* in cultures is possible by evaluating sequence polymorphisms in the IS*1311* gene by PCR and REA and is the most common method used in Australia.[23, 192] Finer level typing is possible using RFLP, variable number tandem repeat (VNTR) or PFGE methods, but these require pure cultures.

For the identification of strain type from cultures, conventional IS*1311* PCR primers described by Marsh *et al* (M56, M119, 608 bp target)[23] are recommended. REA of the IS*1311* PCR product with enzymes *Hinf*I and *Mse*I is required to identify the polymorphism associated with different types from *Map* cultures. *Map* strains can be separated into C, S and B (Bison) types, based on cytosine/thymidine (C/T) polymorphisms at base pair 223. At that site, the presence of cytosine and thymidine nucleotides occurs in C strains, only cytosine nucleotides in S strains and only thymidine nucleotides in B strains.[23, 24, 192] Following REA, *Map* C strains are characterized by four gel bands at 67, 218, 285 and 323 bp, while S strains show two bands of 285 and 323 bp and B strains show three bands of 67, 218 and 323 bp.[24, 193]

Reagents and Materials

Sample preparation as described for the IS*900* PCR.

*Conventional IS*1311 *PCR procedures*

For cultures, the 1311L reaction using primers as described in Table 7 is applied, while for fixed tissues the 1311S reaction is used. These primers and conditions are as described in earlier Australian studies.[23, 24] The primers involve a single forward primer [M56 : GCG TGA GGC TCT GTG GTG AA] and one of the following reverse primers: M94: CAG CGA TCG TCG ACA GTG TG; M119: ATG ACG ACC GCT TGG GAG AC. The conditions are the same as for the 900M PCR. The controls required are shown in Table 8 and are similar to those used for the conventional IS*900* PCR as described earlier.

Table 7. Suggested PCR reactions for application in *Map* REA procedures

Source material	Target	Reaction (name)	Forward Primer	Reverse Primer	Predicted Product (bp)	Reference
Culture	IS*1311*	1311L	M56	M119	608	Marsh et al 1999[23] Whittington et al 2001[24]
Fixed tissue	IS*1311*	1311S	M56	M94	268	Marsh et al 1999[23]

Table 8. Controls required for IS*1311* PCR and REA assays

Control type	PCR controls	REA controls
Positive	Extraction from *Map* positive ovine faeces or tissue	PCR product from extraction positive control
Positive	*Map* cattle strain	PCR product from *Map* cattle strain control
Positive[a]	*Map* bison strain	PCR product from *Map* bison strain control
Positive	-	Uncut PCR product from *Map* cattle strain control (i.e not exposed to enzymes)
Negative	PCR MilliQ water	REA MilliQ water
Negative	PCR cocktail only	REA cocktail only
Negative	Extraction from negative control faeces/tissue	-

[a] Only required where this is a likely outcome

*IS*1311 *PCR REA procedure*

REA can only be performed on IS*1311* amplified products that show a specific gel band (i.e. a positive result). The volume of amplified product required for REA is determined according to the intensity of the observed specific amplified product. As a guide, a volume range for the different PCR reactions is shown in Table 9, where a PCR reaction grade 4+ is a very strong positive gel line, and a 0.5 to trace represents a barely visible line of reaction.

Table 9. Volumes suggested for REA depending on strength of relevant PCR reaction

IS*1311* PCR reaction grade	Volume range suggested for REA (μL)
4+	**1-5**
3+	**2-6**
2+	**5-12**
1+	**10-19.2**
0.5 to trace	**19.2**

The REA cocktail volume is determined by the number of samples plus 1 spare for every 10 tubes. The following tables can be used to calculate the cocktail reagent volumes for IS *1311* REA reactions. When gel bands of 2+ to 4+ are found in the 1311L PCR, routine REA volumes of 16 μL according to Table 10 can be applied, while for 1311S reactions of this strength, routine REA volumes of 16 μL according to Table 11 are applicable. For weak gel bands of up to 1+, reactant volumes need to be increased to 25 μL according to Tables 12 and 13.

Table 10. Reagents for routine REA for 1311L, for a volume of PCR product (X) to a maximum of 12.1 μL

Reagent	Volume per tube (μL)	Final concentration/amount
MQW	Up to final volume	
10 × NE Buffer 2	1.6	1 ×
10 × BSA (1μg/μL)	1.6	1.6 μg
Mse 1 (4 U/μL)	0.5	2 U
Hinf 1 (10 U/μL)	0.2	2 U
Cocktail Volume	16-X	
PCR product	X	
Final Volume	16	

1090

Table 11. Reagents for routine REA for 1311S, for up to 14.2 μL of PCR product

Reagent	Volume per tube (μL)	Final concentration/amount
MQW	Up to FV	
10 × NE buffer 2	1.6	1 ×
Hinf 1 (10 U/μL)	0.2	2 U
Cocktail Volume	16-X	
PCR product	X	
Final Volume	16	

Table 12. Reagents for special REA reaction for 1311L for a volume of PCR product (X) to a maximum of 18.9 μL

Reagent	Volume per tube (μL)	Final concentration/amount
MQW	Up to final volume	
10 × NE buffer 2	2.5	1 ×
10 × BSA (1μg/μL)	2.5	2.5 μg
Mse 1 (4 U/μL)	0.8	3.2 U
Hinf 1 (10 U/μL)	0.35	3.2 U
Cocktail Volume	25-X	
PCR product	X	
Final Volume	25	

Table 13. Reagents for special REA reaction for 1311S for up to 22.2 μL of PCR product

Reagent	Volume per tube (μL)	Final concentration/amount
MQW	Up to final volume	
10 × NE buffer 2	2.5	1 ×
Hinf 1 (10 U/μL)	0.35	3.2 U
Cocktail Volume	25-X	
PCR product	X	
Final Volume	25	

For IS*1311* REA, 4% agarose gel is typically required to accurately determine the results, with appropriate molecular weight markers (Reference ladder: Roche Molecular Weight Markers VIII). Other workers have applied 2% agarose,[193] but this requires longer run times or larger gels. Run the entire volume of digested product (including loading buffer). The predicted REA band sizes for IS*1311* PCR assays 1311L and 1311S are shown in Table 10. Figure 5 shows the expected gel profile for B, C and S strains of *Map* and for *M. avium* subsp *avium* (if required).

*IS*1311 *PCR and REA quality control acceptance criteria*

It is assumed that an IS*900* PCR and REA has been performed prior to IS*1311* reactions, to confirm that *Map* is present.

For the IS*1311* PCR, the negative controls (PCR MQW, PCR cocktail, negative sample control) must show no bands. The positive controls (extraction positive control, cattle strain positive control) will show a single band equivalent to the original target of the PCR assay used.

For the IS*1311* REA, the negative controls (REA MQW, REA cocktail) must show no bands. The positive controls (extraction positive control, cattle strain positive control, uncut cattle strain positive control) will show bands as indicated in Table 14.

Table 14. Predicted band sizes for *Map* IS*1311* REA based on reactions 1311L and 1311S. The uncut cattle strain control should show a single band at 608 or 268 bp depending on the reaction type.

REA reaction	PCR product (bp)	Enzyme	Species (strain)	Predicted REA bands (bp)
1311L	608	*Mse* 1 & *Hinf* 1	*Map* (S type)	285, 323
			Map (C type)	67[a], 218, 285, 323
			Map (B type)	67[b], 218, 323
			M.a. avium[c]	134, 189, 285
1311S	268	*Hinf* 1	*Map* (S type)	268
			Map (C type)	50, 218, 268
			M.a. avium[c]	268

[a] faint band ; [b] faint band but stronger than C strain; [c] if applicable

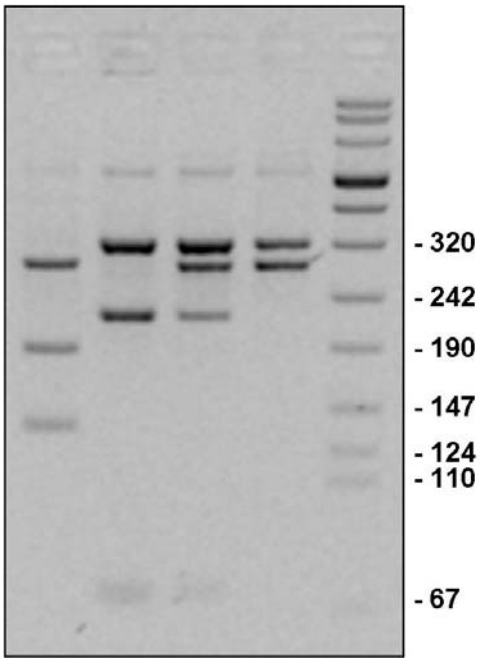

Figure 5. IS*1311* REA profiles (1311L) for *Map* B, C and S strains and for *M. avium* subsp *avium*
Lane 1 Consistent with *M. avium* subsp. *avium*
Lane 2 Consistent with *Map,* bison strain
Lane 3 Consistent with *Map*, cattle strain
Lane 4 Consistent with *Map*, sheep strain
Lane 5: Molecular weight markers

Interpretation of Results

Culture on solid media

Cultures with colonies of typical appearance that are mycobactin dependent are considered 'consistent with *Map'*. If the colonies are demonstrated to contain IS*900* on PCR/REA testing then the sample is considered 'culture positive for *Map'*.

Cultures with no growth after the prescribed incubation period are considered negative on solid media culture. Where overgrowth of irrelevant microbes on solid media renders cultures uninterpretable, the culture is considered to be 'contaminated'. This is equivalent to an inconclusive test outcome.

Liquid culture in M7H9C media

Liquid cultures may be subcultured to solid media to examine colony morphology and mycobactin dependence, or to obtain purified cultures for further study or storage. In ovine pooled faecal samples, a false negative culture rate of 10-25% can be encountered on subculture, reflecting the lower sensitivity of solid medium subculture compared with PCR of primary liquid cultures.[137] The overall contamination rate of pooled and individual fecal culture with liquid media in Victoria was about 5-7% (JM Gwozdz, personal communication 2013). In NSW the contamination rate (presence of irrelevant microorganisms in liquid cultures with or without *Map*) was 7% for ovine faecal culture and <0.2% for tissue culture.[137]

Isolates subcultured onto solid media from a sample of liquid culture that have typical colony appearance and are mycobactin dependent, are considered 'consistent with *Map'*. If this sample is also shown to contain IS*900* on PCR/REA testing (in either M7H9C broth or from

solid media) then the sample is considered 'culture positive for *Map'*.

Where growth in liquid media is identified as being due to organisms other than *Map*, or where overgrowth of irrelevant microbes on solid media renders cultures uninterpretable, the culture is considered to be contaminated.

Liquid cultures that are positive for IS*900* on PCR/REA testing are considered to have 'DNA consistent with *Map'*. Growth of irrelevant microbes on solid media does not negate a finding from liquid media of DNA consistent with *Map*.

Liquid cultures that are negative for IS*900* on PCR/REA are considered as 'no *Map* detected'.

Liquid cultures that are negative for IS*900* on PCR/REA and produce no growth on solid media subculture are considered as 'negative'.

Liquid cultures that are negative for IS*900* on PCR/REA testing and produce growth of irrelevant microbes on solid media subculture are considered to be 'no *Map* detected, contaminated'.

High Throughput Johne's (HT-J) Direct PCR Assay

Principle of the Test

The HT-J direct PCR assay has been developed by the University of Sydney and EMAI to detect *Map* DNA in faeces. It is based on a semi-automated DNA extraction system that utilises bead beating and a commercial DNA extraction kit designed for robotic workstations, followed by qPCR procedures that utilise primers to detect the IS*900* target and melt curve analysis based on SYBR green chemistry. The test has been validated for sensitivity and specificity with bovine and ovine faecal samples on two qPCR platforms (ABI 7500, Stratagene Mx3000). As a DNA-based assay, it does not rely on viable *Map* for detection, but storage of samples at -80ºC is critical where processing cannot be undertaken on receipt at the laboratory. The test is based on primers and amplicon described by Kawaji et al (2007),[118] modified and validated by scientists at the University of Sydney and EMAI.[119]

Concurrent pooled faecal culture and HT-J assay

If it is desired to prepare an homogenised pooled faecal sample for testing by direct faecal PCR using the HT-J protocol, refer to 'Homogenisation of pooled faeces' in the culture section for detailed methods of pooling for various species. The volume of faeces/faecal suspension that is transferred to the HT-J assay is dependent on the faecal pooling method used. In general, if an homogenate is generated using a blender without the addition of saline (Method 1 under 'Homogenisation of pooled faeces'), this material can be treated as for an individual faecal sample at Step 6 of the HT-J protocol. If an homogenate is generated with the addition of saline using a stomacher method (Methods 2.1, 2.2 and 2.3 under 'Homogenisation of pooled faeces'), this material is added post-sedimentation at Step 9 of the HT-J protocol. The appropriate volume to add for each method variant is shown in Table 15.

Table 15. Calculation of the equivalent volume of faecal suspension to transfer post-sedimentation into the HT-J assay for each pooling method variant described in the 'Culture' section under "Homogenisation of pooled faeces".

Pooling method variant	Volume of pooled faecal homogenate	Volume of saline for sedimentation	Equivalent amount of faeces	Volume to be transferred after sedimentation (Step 9 of HT-J protocol)
Method 1	Refer to protocol	-	-	-
Method 2.1	3-4 mL	10 mL	1.5 – 2.0 g	3.5 mL
Method 2.2	3-4 mL	10 mL	1.5 – 2.0 g	3.5 mL
Method 2.3	10 mL	10 mL	1.67 g	5-6 mL

Note 1: *For pooling Method 1, the homogenised faecal sample may be treated the same as an individual faecal sample for the purposes of input volumes at Step 6 of the HT-J protocol.*
Note 2: *The volumes in columns 2, 3 and 4 are derived from earlier described culture methods (refer Figure 2). The volumes described in column 5 are chosen for equivalence to the amount of faecal substrate for HT-J that would be present in 4 mL of supernatant from Method 1 (described at Step 9 of the HT-J procedure). Minimal variation from these specified volumes are recommended for test standardisation.*

The full HT-J assay protocol includes: Part 1. HT-J faecal DNA extraction procedure (that incorporates the Qiagen Biosprint®96 One-For-All Vet kit), and, Part 2. HT-J qPCR procedure.

Reagents and Materials

Reagents (including suggested suppliers)

- Sterile saline (0.85% w/v)
- 100% ethanol (molecular grade)
- Isopropanol (molecular grade)
- BioSprint®96 One-For-All Vet, Qiagen (Cat no. 947057)
- SensiMix SYBR Low-ROX Kit, Bioline (Cat nos. QT625-02 [250 Reactions], QT625-05 [500 Reactions], QT625-20 [2000 Reactions])
- Water, nuclease free (molecular grade) (Sigma, Cat no.W4502)
- PCR Primers (Product size 183bp)[118]
  MP10-1: 5'-ATG CGC CAC GAC TTG CAG CCT -3'
  MP11-1: 5'-GGC ACG GCT CTT GTT GTA GTC G-3'

Disposables

- Plastic transfer pipette, sterile
- 1.5mL flip top tube (e.g. DNA Lo-Bind tubes 1.5mL, Eppendorf)
- 2.0 mL bead tube (Sarstedt, Cat no. 72.694.006)
- 10 mL centrifuge tube (Sarstedt, Cat no. 62.9924.284)
- 15 mL faecal tube (EasyFlip Centrifuge Tubes, JETBIOFIL, Cat no. CFT-212-150; Sarstedt, Cat no. 80.623.111)
- 0.1 mm Zirconia/Silica Beads (BioSpec Products Inc, Daintree Scientific, Cat no. 11079101z)
- P1000 tip (ART, Cat no. 2079E)
- P1000 tip Reach (ART, Cat no. 2079)
- P1000 tip wide bore (ART, Cat no. 2079G)
- 0.2 mL individually-capped PCR tubes (Scientific Specialties Inc. 3240-09)

- 96 well Real time PCR plate (Applied Biosystems, Cat no. 4346906)[a]
- 96 well Real time plate cover (Applied Biosystems, Cat no. 4311971)[a]

[a] If using an alternative validated platform (e.g. Stratagene), appropriate plates and covers should be used)

Equipment

- Pipettes covering a range of 10 µL to 1000 µL
- 25-1,250 µL multichannel
- Top pan balance
- Vortex machine
- Centrifuge with spin capability of 1,231 ***g***
- Microfuge with spin capability of 16,060 ***g***
- Mechanical cell disruption (bead beating) machine (e.g. Mini-beadbeater-96, Biospec Products; Fast Prep® 24, MP Biomedicals; TissueLyser II, Qiagen)

Platforms

- Automated magnetic particle processor (eg. KingFisher™ Flex, Thermo Scientific; MAGMAX™ Express 96, Ambion; BioSprint® 96 Workstation, Qiagen)
  Note: The magnetic particle processor program script (BS96 Vet 100) for the BioSprint® 96 One-For-All Vet protocol can be obtained from Qiagen technical help and is able to be run by all of the above instruments.
- Magnetic head suitable for deep well plates (available from supplier of magnetic particle processor if not standard)
- Quantitative (real-time) thermocycler

Test Procedures

Specimen collection and handling

Faecal samples are collected in an appropriate sterile sample container by the veterinary practitioner. The samples are stored at 4°C by the veterinary practitioner prior to delivery and on ice whilst in transit to the laboratory. Samples are best sent to the laboratory as soon as possible, avoiding unnecessary delays in transit (e.g. delivered to the laboratory during weekdays avoiding storage over the weekend). On arrival at the laboratory, faecal samples should be either stored at -80°C or mixed thoroughly by hand using a wooden applicator stick and two aliquots removed and placed in sterile containers to enable both culture (if required) and HT-J testing. These aliquots and the remainder of the faecal sample should be stored at -80°C until DNA extraction is to be undertaken.

Part 1: HT-J faecal DNA extraction procedure

Important information regarding the BioSprint® 96 One-For-All Vet kit (Qiagen): This procedure incorporates in part a modification of the 'Purification of Viral Nucleic Acids and Bacterial DNA from Animal Tissue Homogenates, Serum, Plasma, Other Body Fluids,Swabs, and Washes' protocol described in the BioSprint® 96 One-For-All Vet Handbook (Qiagen, August 2009). Significant changes to the input volumes and processing steps for the sample have been made to optimise for *Map* detection in faecal matter. It is critical to follow the modified protocol steps described below, and not the handbook protocol. Sections of this protocol have been adapted from the BioSprint® 96 One-For-All Vet Handbook. Consult the above handbook for safety information, kit contents and storage recommendations, product

use limitations, warranty and quality control.

All steps including centrifugation are performed at room temperature (15–25°C) unless otherwise stated.

1. Key list all samples to be used. Prepare an extraction plan using the key listed samples.
2. Include the following controls for each extraction:
   i. Positive control faeces from an infected animal, that produces a positive faecal culture result
   ii. Negative control faeces from a non-infected animal, that gives a negative faecal culture result
   iii. Process control, containing no faeces, only the buffers in the extraction process
3. Prepare reagents from the BioSprint® 96 One-For-All Vet kit as follows:
   a. Buffer AW1. Supplied as a concentrate. Add 160 mL ethanol to the bottle. Tick to show ethanol has been added and record date. Reconstituted Buffer AW1 can be stored at room temperature for up to 1 year.
   b. Buffer RPE. Supplied as a concentrate. Add 220 mL ethanol to the bottle. Tick to show ethanol has been added and record date. Store reconstituted Buffer RPE at room temperature.
   c. Carrier RNA stock solution. Add 310 µL of Buffer AVE to the carrier RNA tube (310 µg) to obtain a final concentration of 1 µg/mL. Store at -20°C in aliquots. Do not freeze-thaw the aliquots of carrier RNA more than three times.
   d. Lysis/Binding solution
      i. Check Buffer RLT has not formed a precipitate during storage. If it has, warm to re-dissolve (37°C) then return to room temperature.
      ii. Important: Do not add Isopropanol or MagAttract Suspension G to Buffer RLT mixture at this stage.
      iii. Prepare Buffer RLT Lysis/binding solution as shown in Table 16. This should be prepared fresh on the day of the extraction.

Table 16. Lysis/binding solution preparation

Reagent	Number of samples[a]		
	1	48[b]	96[b]
Buffer RLT	597 µL	31 mL	59.7 mL
Carrier RNA[c]	2.8 µL	146 µL	280 µL
TOTAL	600 µL/tube		

[a] The volume prepared allows for pipetting error.
[b] For sample numbers (x) other than 48 or 96, calculate the required volumes by multiplying volumes for 1 sample by (x + 4)

4. [c] Carrier RNA does not dissolve in Buffer RLT and must first be dissolved in Buffer AVE before addition to Buffer RLT mixture (Step 3c)For each sample and control to be used, fill a 2.0 mL conical base screw capped bead beating tube with 0.3 g of zirconia/silica beads. These will be used during the bead beating step later in the process.
5. Add 10 mL of sterile saline (0.85% w/v) to a 15 mL faecal tube for each sample and control.
6. Using a balance, weigh and add faeces to the 15 mL faecal tube with the supplied sterile spoon. Discard the spoon.

a. Individual faeces - 1.2 g dry faeces or 1.5 g moist faeces
b. Pooled faeces – If the homogenised faecal pool is prepared without the addition of saline, weigh 1.2 g dry faeces or 1.5 g moist faeces. For all other pooling methods, refer to the section 'Concurrent pooled faecal culture and HT-J assay' above.

7. Thoroughly mix the faeces by shaking the tube vigorously.
8. Allow the faeces to settle for 30 minutes, give the suspension a gentle flick after 5 minutes to release any air bubbles and dislodge any floating debris.
9. Transfer 3 to 5 mL of the top portion of the supernatant to a 10 mL centrifuge tube using a sterile plastic transfer pipette. For pooled faeces see 'Concurrent pooled faecal culture and HT-J assay' and Table 15 at the start of this section for the appropriate volume of supernatant to transfer post-sedimentation to a 10 mL centrifuge tube.
10. Centrifuge at 1,231 ***g*** for 30 minutes with low brake then carefully pour off and discard the supernatant without dislodging the pellet.
11. Remove any remaining supernatant with a pipette and P1000 aerosol resistant tip.
12. Add 600 µL of Lysis/binding solution (see step 3d) to the pellet with a P1000G (wide bore) aerosol resistant tip.

a. Without touching the wall of the tube with the shaft of the pipette, re-suspend the pellet by pipetting up and down and transfer the full volume of Lysis/binding solution and pellet to a prepared bead tube (see step 4).
b. Alternatively, using a sterile transfer pipette, re-suspend the pellet by gently pipetting up and down, keeping the sample in the lower portion of the transfer pipette and not allowing it to lodge in the bulb, and transfer the full volume of Lysis/binding solution and pellet to a prepared bead tube (see step 4).

13. Place bead tubes into mechanical cell disruptor (bead beater) and run:
    a. at the maximum speed (36 oscillations/second) for 90 seconds in a frozen (-80ºC) 1.5 mL tube holder using a Mini-Beadbeater-96 bead-beater, or,
    b. at the maximum speed (6.5 m/second) for 60 seconds, twice, using a Fast Prep-24 bead-beater, or,
    c. at 30 oscillations/second for 100 seconds, using a Tissuelyser II.
14. Centrifuge at 16000 ***g*** for 3 minutes in a microfuge.
15. Transfer all the supernatant (~600 µL) to a new flip top 1.5 mL tube.
16. Centrifuge at 16000 ***g*** for 3 minutes. (The supernatant will be transferred to a plate in Step 20)
17. Set up for magnetic bead purification method. Check that buffers (Buffer AW1, Buffer RPE) have been prepared (see steps 3a and 3b)**.** Label four deep 96 well (termed S-Block) plates and one standard 96 well plate, according to Table 17.
18. Aliquot the appropriate buffers/volumes into the Elution plate and Wash plates (Wash 1, 2 and 3), according to Table 17.
    a. Mix the reconstituted Buffer AW1 and Buffer RPE before use by shaking the bottle

5 times

b. For pipetting with multichannel pipettes, pour buffer into a reagent container provided with the kit
In each plate, the number of wells filled with buffer should match the number of samples + controls to be processed; e.g. if processing 48 samples + controls, fill 48 wells per plate. Ensure that the buffers are added to the same positions (eg. A1, A2 …) in each plate. Refer to plate plan (see step 1).

Table 17. Plate plan, reagent volumes and magnetic processor loading order/position

Load position	Plate name (loading message)	Plate type	Reagent to add	Volume per well (µL)
6	Rod cover[a]	Standard 96 well[a]	-	-
5	Elution	Standard 96 well	Buffer AVE	75
4	Wash 3	S-block [a]	Buffer RPE	500
3	Wash 2	S-block	Buffer RPE	500
2	Wash 1	S-block	Buffer AW1	700
1	Lysate	S-block	Lysate	See step 20

[a] Rod cover, Standard 96 well and S-block (deep well) plates are supplied in the BioSprint® 96 One-For-All Vet kit

19. Prepare the Bead Mix according to Table 18. Important: Before adding MagAttract Suspension G, ensure that it is fully re-suspended. Before the first use, shake the bottle, and vortex for 3 minutes. Before subsequent uses, shake the bottle and vortex for 1 minute.

Table 18: Bead mix preparation

Reagent	Number of samples[a]		
	1	**48**[b]	**96**[b]
Isopropanol	300 µL	14.4 mL	28.8 mL
MagAttract Suspension G[c]	25 µL	1.2 µL	2.4 mL
TOTAL	Add only 300 µL/well		

[a] The volume prepared allows for pipetting error.
[b] For sample numbers (*x*) other than 48 or 96, calculate the required volumes by multiplying volumes for 1 sample by (*x)*
[c] MagAttract Suspension G must be re-suspended by vortexing before addition to Isopropanol (see Step 19-'Important').

20. Load the Lysate plate, referring to the plate plan (see Step 1)

a. Pipette 40 µL of Proteinase K into the bottom of the appropriate wells of the lysate (S-Block) plate.
b. For each sample, transfer as close to 400 µL as possible of the supernatant (from Step 16) with a pipette and P1000 aerosol resistant tip to the lysate plate, without disturbing any pellet.
c. Vortex the prepared Bead Mix (Table 18) thoroughly for 30 seconds immediately prior to aliquoting.
d. For pipetting with a multichannel pipette, pour the mix into a reagent container provided with the kit.

e. Add 300 µL of Bead Mix to all wells according to the plate plan.

Note: In general, it is not necessary to vortex the mixture during dispensing if working without interruption. If dispensing takes longer than 3 minutes per 96-well plate, seal the reagent container tightly and vortex carefully to ensure that MagAttract Suspension G remains fully re-suspended.

21. Place one deep well Rod cover (supplied) into a standard 96 well plate (Table 17, Load position 6).

22. Switch on the automated magnetic particle processor (KingFisher/ MAGMAX/ BioSprint 96) at the power switch. Slide open the front door of the protective cover.

23. Select the "BS96 Vet 100" protocol using the up and down keys on the front panel. (The program script for the protocol is available from Qiagen(www.qiagen.com). Refer to the instrument manual for details on loading the program.)

24. Press 'Start' to start the protocol run.

25. Load plates into the magnetic particle processor (refer also to Table 17):

a. The LCD displays a message requesting the operator to load slot 6 of the worktable inside the instrument (which should be directly adjacent to the door) with the 96-well rod cover.

b. After loading slot 6, press 'Start'. The worktable will rotate, and a new message appears, requesting the operator to load slot 5 with the Elution plate. Load slot 5 and press 'Start' again.

c. Ensure the Elution plate and all subsequent plates are loaded onto the worktable so that well A1 on the plate is aligned with the plate slot label (i.e. well A1 faces inward).

d. Continue this process of pressing 'Start' and loading the particular slot until all slots are loaded.

26. After loading the last slot (Lysate), slide the door shut to protect samples from contamination.

27. Press 'Start' to start sample processing.

28. After the samples are processed, remove the plates as instructed by the display of the instrument. Press 'Start' after removing each plate. The first plate to be removed will be the Elution plate, containing the purified DNA samples.

29. Press 'Stop' after all plates are removed and switch off the instrument. (See Safety/bio-safety precautions/special laboratory requirements for waste disposal)

30. Transfer the contents of individual wells of the Elution plate to appropriately-labelled 200 µL PCR tubes. Store at 4ºC if PCR to be performed within 24 hours or at -20ºC/-80ºC.

Part 2: HT-J qPCR procedure

31. Prepare a qPCR worksheet to accommodate samples to be tested.

32. Include the following controls for each qPCR run:

i. Positive control. Purified DNA from *Map* used to produce a 10-fold dilution series

(see Step 33 below)

ii. Negative control. Contains PCR buffers and purified sterile water used to produce the DNA standards.

iii. qPCR process control (or No Template Control). Contains qPCR cocktail reagents only.

33. Prepare *Map* genomic DNA standards purchased from the University of Sydney as per product insert and validate these against reference information supplied in the product insert. Information from the product insert is also available in Part 3 of this ANZSDP.

Note*:* Up to four replicates of standard 5 should be included to increase the chance that at least two react.

34. Calculate volumes of qPCR mastermix required by referring to Table 19. For each faecal sample, DNA extracts are tested in duplicate (replicates 1 and 2).

Table 19. Reagents and volumes to prepare qPCR mastermix

Ingredient	Stock Concentration or Amount	Final Concentration	Number of PCR assays[a]	
			1	96
2 x SensiMix SYBR Low-ROX	2×	1×	12.5 µL	1.25 mL
Water (molecular grade)	-	-	7.25 µL	725 µL
Primer MP10-1	50 µM	250 nM	0.125 µL	12.5 µL
Primer MP11-1	50 µM	250 nM	0.125 µL	12.5 µL
Final volume			20.0 µL	2 mL
Template DNA			5.0 µL	-

[a] Volumes are given in µL for 1 or 96 assays. For volumes required for (*x*) assays, multiply volumes for 1 PCR assay by (*x*+4).

35. Aliquot mastermix into the qPCR reaction plate in accordance with good nucleic acid testing laboratory guidelines.
36. For all samples and controls, add 5 µL of template DNA/control to PCR reaction plate.
37. Load plate into thermocycler and run using the thermocycling parameters described in Table 20.

Table 20. Thermocycling parameters (ABI 7500 thermocycler; Stratagene MX300)

Stage	Temperature (ºC)	Time	Cycles
Initial denaturation	95	10 mins	1
Denaturation	95	15 secs	
Annealing	68	30 secs	40
Extension	72	1 min	
Melt Curve	65-95		1

38. Analyse results following test acceptance criteria (see *Interpretation of Results* below).

Safety/biosafety precautions

Refer to the BioSprint® 96 One-For-All Vet Handbook for chemical safety information regarding kit reagents. After running the automated magnetic-bead program, the liquid waste solutions from Plates A to D should be transferred to an appropriately labelled hazardous waste container for disposal. The empty 96 well plates can then be discarded in biological hazard waste bins.

Technical and professional staff requirements

Staff undertaking these procedures must be qualified by an appropriate TAFE or university certificate, diploma or degree and have experience and knowledge in the procedures of DNA extraction from clinical samples and quantitative real-time PCR set up and dynamics.

All staff should have training and experience in, knowledge of and ability to follow QA procedures, especially those procedures and practices required to minimise false positive reactions from amplicon contamination.

Appropriately qualified technical and professional staff must be trained and deemed to be experienced and competent in all aspects of the entire HT-J assay procedure including:

- Sample handling and storage
- Sample and assay documentation
- DNA extraction
- Quantitative real-time PCR
- Data analysis and result recording
- Reporting.

Interpretation of Results

For a valid set of results each qPCR run must meet the following acceptance criteria:

- a standard curve derived from standards 1-4 or 5 producing an amplification efficiency between 90.0 and 110.0% and controls producing the correct outcome. While uncommon, an obvious outlier of a single replicate within standards 1-4 can be removed, using professional judgement.
- at least one positive result from up to four replicates of standard 5.
- positive and negative extraction and qPCR controls in each PCR run meet the criteria shown in Table 21.

To be positive each replicate of the positive controls and standards must have:

- based on the recommended mastermix (Sensimix SYBR Low-ROX Kit Bioline), a melting temperature (T_m) of 89.4+1.5^{o}C (Stratagene platform) or 87.8+1.5^{o}C (ABI platform), and
- a DNA quantity ≥ the cut-point of 0.001 pg (1/5 genome equivalent) as determined by a standard curve derived from the standards included in each qPCR run.

Table 21. Positive and negative control acceptance criteria

Procedure	Control	Acceptance criteria
Extraction	Positive faeces (refer Part1, Step 2)	Positive
	Negative faeces (refer Part1, Step 2)	Negative
	Extraction process control[a] (refer Part1, Step 2)	Negative
	Extraction plate control[b]	Negative

qPCR	Positive (refer Part 2, Step 32)	Positive
	Negative (refer Part 2, Step 32)	Negative
	qPCR process control (cocktail only) (refer Part 2, Step 32)	Negative

[a] Saline + buffers; [b] Buffer RLT only in place of sample lysate in extraction plate.

For each faecal sample, DNA extracts are tested in duplicate (replicates 1 and 2). For an individual DNA extract to be graded as positive in the qPCR assay, the following criteria must be met:

- Based on the recommended mastermix (SensiMix SYBR Low-ROX Kit, Bioline), a T_m of 89.4+1.5°C (Stratagene platform) or 87.8+1.5°C (ABI platform).
- For replicates with T_m's within the correct range, the mean DNA quantity of the 2 replicates is ≥ the cut-point of 0.001 pg (1/5 genome equivalent) as determined by a standard curve derived from the standards included in each qPCR run.

Note 1: If one or both replicates have a T_m that is not within the correct range, any DNA quantity recorded in that reaction is not MAP-specific and should be disregarded, that is, the value should be corrected to 'zero'.

Note 2: Professional judgment should be used to determine when a sample should be retested, for example where one replicate has no C_T and the other a low C_T (consistent with pipetting error).

A POSITIVE result:	To be considered positive, the mean DNA quantity of replicates 1 and 2 must be positive.
A NEGATIVE result:	To be considered negative, the mean DNA quantity of replicates 1 and 2 must be negative.

Note 3: With regard to the acceptable T_m range using other qPCR platforms (such as the Rotorgene platform) and/or mastermixes, this would need to be determined and fully validated using known positive faecal samples (faeces from animals/pooled samples derived from an infected herd/flock, previously examined by faecal culture and having a positive faecal culture result).

An estimate of this acceptable range can be derived based on the T_m results for the *Map* genomic DNA standards that are included in the qPCR. For the University of Sydney and EMAI data used in test validation, the standards have an average T_m that approximates the centre of the acceptable T_m range. Therefore, to estimate a T_m range, calculate as: T_m range = (Average T_m of *Map* genomic DNA standards) ± 1.5°C.

Note 4: If primer-dimer is observed, its T_m is at least 5°C less than that of the *Map*-specific T_m.

Throughput and turn-around times

Samples are set up for batch testing depending on the capability of the testing laboratory. Samples should be stored at -80°C until the required number for a batch is accumulated. The minimum turn-around time for a sample is 3-4 business days.

Immunological Tests

There is a prolonged delay between infection with *Map* 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 (ELISA)

Principle of the Test

The sensitivity of ELISA is comparable with that of the 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.[194]

Reagents and Materials

Three commercial absorbed ELISA kits are currently available in Australia: Idexx Paratuberculosis Screening Ab test, Parachek® 2 from Prionics, and ID Screen® Paratuberculosis Indirect from IDVET. All kits employ anti-ruminant conjugates that can be applied to bovine, caprine and ovine sera. Only the Idexx Paratuberculosis Screening Ab is approved by SCAHLS, for use in cattle. This test is also equivalent to an earlier Pourquier ELISA that was validated on sera from Australian sheep and goats.[195] A non-absorbed ELISA has also been used in deer in New Zealand,[196, 197] but has not been approved by SCAHLS.

Test Procedure

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 *Map* 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.[103] The overall sensitivity for all infection stages and age-groups of cattle was calculated to be about 15%.[84, 103]

The three commercial ELISA kits available in Australia have specificities above 99%.

All available commercial kits offer an option of testing bovine milk samples, while the Parachek® 2 assay has been applied to ovine and caprine milk. 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.[198, 199]

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,[200-202] but a recent study confirmed that the Idexx (formerly Pourquier) screening ELISA is significantly more sensitive than AGID in both sheep and goats.[195] The reported specificity and sensitivity of the AGID measured against histological results were 99-100% (95% CI) and 38-56% (95% CI), respectively,[200] while specificity in disease-free populations of sheep and goats is estimated to be 100%.[195]

Reagents and Materials

The antigen for this test is prepared from *Map* grown on Watson-Reid media. 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_3BO_3), 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. If weak precipitin lines are observed, plates can be incubated for a further 24 hours, or the test repeated. A modified gel pattern with a larger gap between the antigen and serum wells may assist interpretation of some doubtful reactions. 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 316V 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 (CFT)

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 *Map*.[203]

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/Procedures/Documents/ASDTs/bovine_brucellosis2.pdf).

Test Procedure

Sera are heated at 58ºC (cattle) or 60ºC (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 Sigma-Aldrich. 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.

Primary and secondary incubations are performed at 37ºC 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.

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 CMI

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.[204-210]

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).[211] 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.

Test Procedure

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.[212] 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.[213] The gamma interferon assay is unreliable in calves under 12 months of age due to the occurrence of many false positive results.[214-216]

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 purified protein derivative (PPD) or *M. avium* PPD (25,000 units/mL, AsureQuality, New Zealand). Johnin PPD is not currently available from CSL Limited 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.

Interpretation of Results

An increase in skin thickness of >3 mm is taken as positive. 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.[209] Furthermore, the interpretation of the skin test results is complicated by the lack of agreement with respect to interpretation criteria.

In a 2003 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 ≥2 mm, 91.3% at the cut-off value of ≥3 mm and 93.5% at the cut-off value of ≥4 mm.[212] 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.[212]

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 Condron, GJ Eamens and RJ Whittington.

The current edition incorporates advances in knowledge, technology and standardisation since the previous revisions performed in 2002 by DV Cousins, RJ Condron, GJ Eamens, RJ Whittington and GW de Lisle and in 2010 by JM Gwozdz. It was written with the assistance of R McCoy, Gribbles South Australia, and E Sergeant, AusVet Animal Health Services, Orange NSW.

Part 3 – Reagents and Kits

Ziehl-Neelsen stain

1. Ziehl-Neelsen carbol fuchsin

Basic fuchsin	0.3 g
Ethanol (95% v/v)	10 mL
Phenol	5.0 g
Distilled water	95 mL

2. Acid alcohol

Ethanol (95% v/v)	97 mL

	Concentrated HCl	3 mL
3.	Counterstain	
	Malachite green or Methylene blue	1.0 g*
	Distilled water	100 mL
4.	Alkaline 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.

* Lower concentrations of methylene blue chloride (0.3% w/v) are also satisfactory.

1790

Kinyoun acid fast stain

1.	Kinyoun carbol fuchsin		
	Solution A:	Basic fuchsin	4 g
		Ethanol (95% v/v)	20 mL
	Solution B:	Phenol (melted crystals)	8.0 g
		Distilled water	100 mL
	Mix solutions A and B and stand for 2-3 days before use.		
2.	Acid alcohol		
1800	Ethanol (95% v/v)		97 mL
	Concentrated HCl		3 mL
3.	Counterstain		
	Methylene blue		0.3 g
	(or Brilliant green		1 g)
	(or Malachite green		0.5 g)
	Distilled or deionised water		100 mL

1810

Culture Media and Reagents
Decontamination reagents

1820

HPC/BHI

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

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

Mix and dissolve (heating at 58ºC may be required) then autoclave at 120ºC 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. When 0.9% w/v HPC is diluted with faeces/saline at a rate of 1:5 (e.g. 4 mL faeces/saline in 20 mL HPC solution, or 5 mL in 25 mL HPC solution), this produces a final concentration of 0.75% HPC. If other concentrations of HPC are required, adjust the amount of HPC in the recipe above.

VAN

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

Note: Avoid skin 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 (10 mg/mL)	1 mL

Mix ingredients then dispense and store at -20°C. For VAN/BHI, add antibiotics to sterile half-strength Brain Heart Infusion instead of water.

Amphotericin B stock solution (10 mg/mL)

Amphotericin B 45% with sodium deoxycholate 35% (Sigma)	50 mg
MilliQ purified water	5 mL

Mix to dissolve, dispense into aliquots and store at - 20°C.

M7H9C
(Modified Middlebrook 7H9 liquid culture medium, University of Sydney, Camden)[143]

This medium can be manufactured from a combination of commercial broth base and purchased additives, based on a medium made up of Middlebrook 7H9 broth with added Casitone, and a supplement containing ADC enrichment, egg yolk, mycobactin J and antibiotics.

For a batch size of 540 mL of final medium (approx 90 tubes):

Part 1: Base

Middlebrook Difco 7H9 broth (BD)	1.68 g
Casitone (Bacto)	0.36 g
Purified water[a]	409 mL

[a] Addition of glycerol (0.2% v/v) or polysorbate 80 (Tween 80; 0.05% w/v) to water is optional for Middlebrook 7H9 medium for mycobacteria but was not included in the formulation of Bactec 12B media.

Autoclave base at 121ºC for 10 minutes, and add supplement when cooled to 45ºC.

Part 2: Supplement

For each 409 mL of base add 131.4 mL of supplement made up as:

BBL Middlebrook ADC Enrichment (BD)	14.4 mL
Egg yolk (approx 13 large eggs)	90 mL
Mycobactin J (50 µg/mL)	9 mL
PANTA antibiotics[b] (BD) (3 vials)	18 mL

[b] Original Bactec 12B medium was supplemented with PANTA PLUS® which is no longer commercially available. PANTA antibiotics (for MGIT system) are available and contain the same antibiotic mixture (as lyophilized antibiotics) reconstituted using 6 mL sterile water per vial

The complete M7H9C medium is aliquoted aseptically in 6 mL volumes into sterile glass or plastic tubes with screw tops.

Modified 7H10 media with Mycobactin J [102]

This media is for culture of sheep or cattle strains of *Map*

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

Autoclave at 121^{o}C for 15 minutes; cool to 58^{o}C.

Using aseptic technique, combine the following additional ingredients and add to the 7H10 base:

PANTA	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^{o}C for 1 week. Perform a suitability check by inoculation of *Map* to demonstrate media ability to support growth of the target organism. Store media at 4^{o}C.

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

This media is for culture of cattle strains of *Map*.

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 121ºC 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.

Media supplements

Mycobactin J

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

For each vial of 2 mg Mycobactin J,[c] add 1 mL ethanol (95% v/v) and 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.

[c] Allied Monitor, 201 Golden Drive, Fayette, Missouri, USA 65248

Malachite green

Prepare a 2% (w/v) 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 halves. Using a 10 mL sterile syringe, extract yolk from the yolk sac and dispense/measure into a 50 mL sterile tube (Falcon). 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 37ºC for 48 hours.

PANTA

PANTA can be reconstituted by adding sterile water to lyophilised PANTA supplement.

Two commercial PANTA supplements are available from BD:

1.	MGIT PANTA	Catalogue 245114	Pack size 6 x 6 mL
2.	PANTA/F	Catalogue 442188	Pack size 10 x 10 mL

The ingredients of these are equivalent, and only differ in pack size which needs to be taken into account before reconstituting. Either can be used to prepare M7H9C and modified 7H10 media.

Absorbed ELISA

Three commercial absorbed ELISA kits: Parachek® 2 (Prionics), Idexx Paratuberculosis Screening Test, ID Screen® Paratuberculosis Indirect (IDVET) are available in Australia, of which only the Idexx kit is currently SCAHLS approved, for use in cattle. The Idexx Paratuberculosis Screening Test and Parachek® 2 kits have been validated for cattle, sheep and goat sera, while the ID Screen® kit has been validated in cattle and may also be applied to sheep and goat sera. 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 the current Johne's Disease Reference Laboratory.

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 Environment and Primary Industries, Bundoora, 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 can be obtained from EMAI, Menangle, NSW[a] or from AgResearch Wallaceville Animal Research Centre, Upper Hutt, NZ.[b] Control positive sheep and goat sera are available from EMAI, NSW.

[a] mark.turner@dpi.nsw.gov.au Tel: 61 2 46406423

[b] geoffrey.delisle@agresearch.co.nz, Tel: 64 4 9221 30

Gamma interferon assay

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

Map genomic DNA for standard preparation (as used in the HT-J assay)

Reconstitution

Map genomic DNA reagent is supplied by the University of Sydney in freeze-dried form. Reconstitute using 0.5 mL of TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 8.0) and allow the vial to stand at room temperature for 20 minutes. Mix the vial very gently. This will give the concentrated stock at a concentration of 2 mg/L (or 2 ng/µL). It is recommended that this be transferred into 1.5ml DNA LoBind tubes (Eppendorf). Store at -80ºC. Do not repetitively freeze-thaw the concentrated stock; aliquot in a minimum of 50 µL and thaw when preparing a large batch of standards.

Method of preparation of standards

Prepare standard dilutions from the concentrated stock of *Map* genomic DNA:

a. Take an aliquot of 2 ng/µL concentrated *Map* gDNA stock from -80ºC freezer and thaw.

b. Prepare 20 pg/µL working stock by performing a 1/100 dilution with TE buffer e.g. 10 µl of 2 ng/µL concentrated Map gDNA + 990 µL TE buffer.

c. Prepare a 5-point serial dilution (ten-fold) of *Map* gDNA in 1.5 mL LoBind Eppendorf tubes, with nuclease free (NF) water (Table 22). Change tips between each addition and mix well.

Table 22. Preparation of DNA standards for HT-J assay

Standard	Description	Manufacture
1	2 pg/µL, 10 pg/qPCR reaction	Add 100µl of 20 µM working stock + 900 µL nuclease-free (NF) water
2	200 fg/µL, 1 pg/qPCR reaction	Add 100µl of Standard 1 + 900 µL NF
3	20 fg/µL, 0.1 pg/qPCR reaction	Add 100µl of Standard 2 + 900 µL NF
4	2 fg/µL, 0.01 pg/qPCR reaction	Add 100µl of Standard 3 + 900 µL NF
5	0.2 fg/µL, 0.001 pg/qPCR reaction	Add 100µl of Standard 4 + 900 µL NF

d. Aliquot standards (minimum volume 50 µL) and store at -80ºC.

e. Use in qPCR: Add 5 µL of each standard DNA dilution to duplicate qPCR tubes, starting from the lowest to highest concentration. Use of quadruplicate qPCR tubes for standard 5 is recommended.

Method of production (template format only)

Map genomic DNA (*Lot # specified here*) was derived from *Map* (*type C or S and strain identity specified here*). Highly pure genomic DNA was extracted using the method described in Choy et al. (1998).[217] DNA quantity and quality was determined using a Nanodrop spectrophotometer. *Map* gDNA (1 µg) was freeze-dried in 1 mL aliquots and stored at 4°C.

One vial was reconstituted and standards prepared as directed above. These were tested in qPCR and gave an acceptable standard curve with an efficiency of 99.5%.

(Note: Refer to product insert for graphs of amplification plots, dissociation curve and standard curve of the relevant Reagent Lot*)*.

Shelf life and stability

Map genomic DNA freeze dried is stable for at least 4 years at 4°C. The minimum shelf life is 6 months when reconstituted and stored as directed.

Recommendations

The use of DNA LoBind tubes from Eppendorf is based on the experience of the Johne's disease laboratory at the University of Sydney. These gave best standard curve efficiency when diluted standards were stored at -80ºC.

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