

## PARATUBERCULOSIS (Johne's disease)

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### SUMMARY

*Paratuberculosis (Johne's disease) is a chronic enteritis of ruminants caused by Mycobacterium avium subsp. paratuberculosis (M. paratuberculosis) (49).*

**Identification of the agent:** *The diagnosis of paratuberculosis is divided into two parts: the diagnosis of clinical disease and the detection of subclinical infection. The latter is essential for control of the disease at the farm, national or international level.*

*Diagnosis of paratuberculosis is made on clinical grounds confirmed by the demonstration of M. paratuberculosis in the faeces by microscopy, culture, or by the use of DNA probes and the polymerase chain reaction. Diagnosis is made at necropsy by the finding of the pathognomonic lesions of the disease in the intestines, either grossly with the demonstration of typical acid-fast organisms in impression smears of the lesions or histologically, and by isolation of M. paratuberculosis in culture.*

*The detection of subclinical infection depends on the detection of specific antibodies by serology, or culture of M. paratuberculosis from faeces or tissues collected at necropsy, 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.*

*Cultures of M. paratuberculosis may be obtained from faeces or tissues, after treatment to eliminate contaminants, by inoculation into artificial media with and without the specific growth factor – mycobactin – that is essential for the growth of M. paratuberculosis.*

**Serological tests:** *Control of paratuberculosis is difficult because of the prolonged course of infection, the predominantly subclinical nature of the disease and lack of tests for accurate detection of subclinically infected animals.*

*The serological tests commonly used for paratuberculosis in cattle are complement fixation (CF), absorbed enzyme-linked immunosorbent assay (ELISA) and agar gel immunodiffusion (AGID). Sensitivity and specificity are often determined by reference to results of faecal culture, which itself has unknown sensitivity in subclinically infected cattle. When used to confirm diagnosis of paratuberculosis in cows with typical clinical signs, some tests, for example CF and absorbed ELISA, perform very well.*

**Requirements for vaccines and diagnostic biologicals:** *Vaccines for paratuberculosis may be live attenuated or killed bacteria either incorporated with an adjuvant or lyophilised and adjuvanted on reconstitution. Bacterial counting is difficult and bacterial content of vaccines may be based on weight, while vaccine potency may be judged by batch tests for sensitising ability in guinea-pigs.*

*Vaccine safety or abnormal toxicity may also be tested in guinea-pigs.*

*For diagnostic skin tests, Johnin and avian tuberculin are purified protein derivatives (PPD) of a heat-treated culture of M. paratuberculosis or M. avium, respectively. Johnin is standardised for content of PPD by chemical assay and its biological activity is identified in guinea-pigs sensitised with M. paratuberculosis. Avian tuberculin activity is determined in guinea-pigs sensitised with M. avium by comparison with a reference preparation calibrated in international units.*

## A. INTRODUCTION

*Mycobacterium avium* subspecies *paratuberculosis* (*M. paratuberculosis*) is an organism first observed by Johne & Frothingham in 1895. *Mycobacterium avium* subsp. *paratuberculosis* causes paratuberculosis or Johne's disease, an intestinal granulomatous infection. First recognised in cattle, then in sheep and later in goats, paratuberculosis is found most often among domestic and wild ruminants and has a global distribution. The disease has also been reported in horses, pigs, deer and alpaca, and recently in rabbits, stoat, fox and weasel (3, 14). Under natural conditions, the disease in cattle spreads by ingestion of *M. paratuberculosis* from the contaminated environment. The disease persists after the introduction of infected animals. Infection can be spread vertically to the fetus (25) and semen can be infected with the organism (46). The primary source of infection in calves is milk from infected cows or milk that is contaminated with the faeces of diseased cattle.

The identification of *M. paratuberculosis* is based on its mycobactin requirement and its pathogenicity in the host. Mycobactin dependence has long been used as a taxonomic characteristic for *M. paratuberculosis* because most mycobacteria are able to make mycobactin for themselves. *Mycobacterium avium* subsp. *paratuberculosis*, *M. silvaticum* and some primary isolates of *M. avium* lack this capacity, however, and require mycobactin to grow in the laboratory. Thus, the mycobactin requirement is not confined to *M. paratuberculosis*; this characteristic exists to various degrees within the *M. avium* group (48).

Clinical signs of paratuberculosis are a slowly progressive wasting and diarrhoea, which is intermittent at first, becoming progressively more severe until it is constantly present in bovines (13). Diarrhoea is less common in small ruminants.

Early lesions occur in the walls of the small intestine and the draining mesenteric lymph nodes, and infection is confined to these sites at this stage. As the disease progresses, gross lesions occur in the ileum, jejunum, terminal small intestine, caecum and colon, and in the mesenteric lymph nodes. *Mycobacterium avium* subsp. *paratuberculosis* is present in the lesions and, terminally, throughout the body. The intestinal lesions are responsible for a protein leak and a protein malabsorption syndrome, which lead to muscular wasting. Clinical signs usually first appear in young adulthood, but the disease can occur in animals at any age over 1–2 years.

Within a few weeks of infection, a phase of multiplication of *M. paratuberculosis* begins in the walls of the small intestine. Depending on the resistance of the individual, this infection is eliminated or the animal remains infected as a healthy carrier. The proportion of animals in these categories is unknown. A later phase of multiplication of the organisms in a proportion of carriers leads to the extension of lesions, interference with gut metabolism and clinical signs of disease. Subclinical carriers excrete variable numbers of *M. paratuberculosis* in the faeces. In most cases larger numbers of organisms are excreted as clinical disease develops.

Delayed-type hypersensitivity (DTH) is detectable early in the infection and remains present in a proportion of the subclinically infected carriers, but as the disease progresses, DTH wanes and may be absent in clinical cases. Serum antibodies are detectable later than DTH. They may also be present in carriers that have recovered from infection. Serum antibodies are present more constantly and are of higher titre as lesions become more extensive, reflecting the amount of antigen present. In sheep, there may be a serological response that is more likely to be detected in multibacillary than in the paucibacillary form of the disease.

Other mycobacterial diseases and infections, including mammalian and avian tuberculosis, cause DTH and the presence of serum antibodies. It follows therefore that these diseases need to be differentiated from paratuberculosis, both clinically and by the use of specific diagnostic tests. Exposure to environmental saprophytic mycobacteria may also sensitise livestock, resulting in nonspecific DTH reactions.

Animals vaccinated against paratuberculosis develop both DTH and serum antibodies. Vaccination is an aid to the prevention of clinical disease, but does not necessarily prevent infection. It also interferes with programmes for the diagnosis and control of bovine tuberculosis. Thus, if it is necessary to attempt a diagnosis of infection in vaccinates, only tests to detect *M. paratuberculosis* in the faeces can be used (21).

In individual animals, especially from a farm in which the disease has not previously been diagnosed, a tentative clinical diagnosis must be confirmed by laboratory tests. However, a definitive diagnosis may be warranted on clinical grounds alone if the clinical signs are typical and the disease is known to be present in the herd. Confirmation of paratuberculosis depends on the finding of either gross lesions with the demonstration of typical acid-fast organisms in impression smears or microscopic pathognomonic lesions and the isolation in culture of *M. paratuberculosis*.

## B. DIAGNOSTIC TECHNIQUES

To diagnose the presence of paratuberculosis in an individual clinically suspect animal, a number of laboratory tests can be used including: faecal smears, faecal and tissue culture, DNA probes using faeces or tissues, serology, necropsy and histology.

Herd tests to detect subclinical infection are carried out to determine the prevalence of the infection, usually so that control measures can be instituted. As no test is 100% sensitive or specific, control of the disease by the disposal of positive reactors depends on repeated tests at 6-month or yearly intervals over a number of years and the elimination of reactors to serological tests or faecal shedders; the removal of offspring from female reactors is also considered to be prudent. Even these procedures are not always successful without changes in hygiene and livestock management to reduce the transmission of infection within a herd (2).

## 1. Identification of the agent

### a) Necropsy

Paratuberculosis cannot be diagnosed on superficial examination of the intestines for signs of thickening. The intestines should be opened from the duodenum to the rectum to expose the mucosa. There is not always a close correlation between the severity of clinical signs and the extent of intestinal lesions. The mucosa, especially of the terminal ileum, is inspected for pathognomonic thickening and corrugation. Early lesions are seen by holding the intestine up to the light, when discrete plaques can be visualised. Mucosal hyperaemia, erosions and petechiation have been observed in deer with paratuberculosis. The earliest lesions are thickening and cording of lymphatic. The mesenteric lymph nodes are usually enlarged and oedematous. Smears from the affected mucosa and cut surfaces of lymph nodes should be stained by Ziehl-Neelsen's method and examined microscopically for acid-fast organisms that have the morphological characteristics of *M. paratuberculosis*. However, acid-fast organisms are not present in all cases. Diagnosis is therefore best confirmed by the collection of multiple intestinal wall and mesenteric lymph node samples into fixative (10% formol saline) for subsequent histology. Both haematoxylin-and-eosin-stained sections and Ziehl-Neelsen-stained sections should be examined. The typical lesions of paratuberculosis consist of infiltration of the intestinal mucosa, submucosa, Peyer's patches and the cortex of the mesenteric lymph nodes with large macrophages, also known as epithelioid cells, and multinucleate giant cells, in both of which clumps or singly disposed acid-fast bacilli are usually, but not invariably, found.

### b) Bacteriology (microscopy)

Ziehl-Neelsen-stained smears of faeces or intestinal mucosa are examined microscopically. A presumptive diagnosis of paratuberculosis can be made if clumps (three or more organisms) of small (0.5–1.5 µm), strongly acid-fast bacilli are found. The presence of single acid-fast bacilli in the absence of clumps indicates an inconclusive result. The disadvantages of this test are that it does not differentiate among other mycobacterial species and only a small proportion of cases can be confirmed on microscopic examination of a single faecal sample.

### c) Bacteriology (culture)

The isolation of *M. paratuberculosis* from an animal provides the definitive diagnosis of infection with the organism. Although culture is technically difficult and time-consuming to carry out, it is the only test that does not produce false-positive results (100% specificity).

The faecal culture is the best test available for the diagnosis of paratuberculosis in live animals. It is believed that the faecal culture method involving the double incubation method for decontamination of samples and cultivation on solid media detects about 30–40% of infected cattle (56). The faecal culture is able to detect most animals in advanced stages of the disease but identifies only a few animals in early stages of infection (56). It will detect infected animals 6 months or more before they develop clinical signs, and during the clinical stage its sensitivity approaches 100%. The culture of bovine and caprine tissues for *M. paratuberculosis* is more sensitive than histopathological examination.

There are several culture methods, which vary with respect to media and sample processing protocols. The cultivation of *M. paratuberculosis* is always performed using special media supplemented with mycobactin J<sup>1</sup>.

*Mycobacterium avium* subsp. *paratuberculosis* organisms are vastly outnumbered by other bacteria or fungi in faecal and intestinal tissue specimens. The successful isolation of *M. paratuberculosis* from such samples depends on efficient inactivation of these undesirable organisms. The optimal method of decontamination must have the least inhibitory effect on growth of *M. paratuberculosis*. Routine decontamination protocols were shown to decrease the number of *M. paratuberculosis* organisms isolated per sample by about 2.7 log<sub>10</sub> and 3.1 log<sub>10</sub> for faeces and tissues, respectively (34).

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1 Mycobactin can be obtained commercially (mycobactin J) from Allied Monitor, P.O. Box 71, 201 Golden Drive, Fayette, MO 65248, United States of America, or Symbiotic Society, 299 av. Jean Jaurès, 69007 Lyon, France.

There are two basic methods in use for the conventional culture of *M. paratuberculosis* on solid media: the method using oxalic acid and NaOH for decontamination and Löwenstein–Jensen (LJ) medium for growth, and the method using hexadecylpyridinium chloride (HPC) for decontamination in combination with Herrold's egg yolk medium (HEYM) for growth. Both media contain mycobactin. Although it has been published that HEYM supports growth of bovine isolates of *M. paratuberculosis* significantly better than LJ (33), recent studies have shown that certain strains grow better on LJ or Middlebrook media (9).

In addition, there is a technique of radiometric culture where growth in liquid medium BACTEC™ 12B (Middlebrook 7H12) supplemented with egg yolk and mycobactin is measured by the release of radioactive <sup>14</sup>CO<sub>2</sub> from palmitate, as a consequence of bacterial metabolism. This method reduces the time required for results and is considered more sensitive than the conventional culture methods on solid media for the detection of both ovine and bovine strains of *M. paratuberculosis* (11, 58). The decontamination protocol involving double incubation of faecal samples in HPC and mixture of antibiotics may further improve culture sensitivity (11). However, as the BACTEC™ system is radiometrically based, it is not feasible for use in some laboratories and has been phased out in others. The evaluation of the usefulness of alternative culture systems based on liquid media such as MGIT (Becton Dickinson) ESPII (Difco) and MB/BacT Alert (Organon Teknika) that do not use radioactive material for the detection of *M. paratuberculosis* is currently in progress.

Primary colonies of *M. paratuberculosis* on solid media may be expected to appear any time from 5 weeks to 6 months after inoculation (7). Sheep strains, including the uncommon, bright yellow pigmented types, grow less well than cattle strains on commonly used media such as HEYM or LJ, and primary cultures should not be discarded as negative without prolonged incubation. The solid medium Middlebrook 7H10 and liquid medium BACTEC™ 12B both supplemented with egg yolk and mycobactin are excellent for cultivation of ovine strains of *M. paratuberculosis* (58).

Primary colonies of the cattle strain of *M. paratuberculosis* on HEYM are very small, convex (hemispherical), soft, non-mucoid and initially colourless and translucent. Colony size is initially pinpoint, it may remain at 0.25–1 mm, and tend to remain small when colonies are numerous on a slope. Colony margins are round and even, and their surfaces are smooth and glistening. The colonies become bigger more raised, opaque, off-white cream to buff or beige coloured as incubation continues. Older isolated colonies may reach 2 mm. The colonial morphology changes with age from smooth to rough, and from hemispherical to mammilate (7, 47).

On modified 7H10 medium, colonies of the cattle strain are less convex than those on HEYM, especially in aged cultures. They 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 HEYM, those on 7H10 are more difficult to detect. Colonies of the sheep strain of *M. paratuberculosis* on modified 7H10 are convex, soft, moist, glistening, off-white to buff, and very similar to the colour of the media. Colonies are typically between pinpoint and 0.5 mm, but can reach 1 mm, and rarely 1.5 mm if few colonies occur on a slope (7).

Saprophytic mycobacteria may have a similar appearance on either medium but are often evident after 5–7 days (7).

For identification of *M. paratuberculosis*, small inoculum of suspect colonies should be subcultured on the same medium with and without mycobactin, to demonstrate mycobactin dependency. Mycobactin is present in the cell wall of the organism, and heavy inoculum may contain enough mycobactin to support the growth of a mycobactin-independent mycobacterium on medium that contains no mycobactin.

#### o Media

Examples of suitable media are:

##### i) Herrold's egg yolk medium with mycobactin (28)

For 1 litre of medium: 9 g peptone; 4.5 g sodium chloride; 2.7 g beef extract; 27 ml glycerol; 4.1 g sodium pyruvate; 15.3 g agar; 2 mg mycobactin; 870 ml distilled water; six egg yolks (120 ml); and 5.1 ml of a 2% aqueous solution of malachite green. Measure the first six ingredients and dissolve by heating in distilled water. Adjust the pH of the liquid medium to 6.9–7.0 using 4% NaOH, and test to ensure the pH of the solid phase is 7.2–7.3. Add the mycobactin dissolved in 4 ml ethyl alcohol. Autoclave at 121°C for 25 minutes. Cool to 56°C and aseptically add six sterile egg yolks<sup>2</sup> and sterile malachite green solution. Blend gently and dispense into sterile tubes.

2 Use fresh eggs not more than 2 days old from a flock that is not receiving antibiotics. With a brush, scrub the eggs with water containing a detergent. Rinse with water and place the eggs in 70° alcohol for 30 minutes. Dry by inserting between two sterile towels. With sterile rat-tooth forceps, crack one end of the eggshell, making a hole of approximately 10 mm,

It is permissible to add 50 mg chloramphenicol, 100,000 U penicillin and 50 mg amphotericin B.

ii) *Modified Dubos's medium (41)*

For 1 litre of medium: 2.5 g Difco casamino acids; 0.3 g asparagine; 2.5 g anhydrous disodium hydrogen phosphate; 1 g potassium dihydrogen phosphate; 1.5 g sodium citrate; 0.6 g crystalline magnesium sulphate; 25 ml glycerol; 50 ml of a 1% solution of Tween 80; and 15 g agar. Dissolve each salt in distilled water with minimum heat and make up to 800 ml. Add mycobactin in alcoholic solution at 0.05% (2 mg dissolved in 4 ml ethyl alcohol), heat the medium to 100°C by free-steaming, and then sterilise by autoclaving at 115°C for 15 minutes. Cool to 56°C in a water bath, add antibiotics (100,000 U penicillin; 50 mg chloramphenicol; and 50 mg amphotericin B) and serum (200 ml of bovine serum sterilised by filtering through a Seitz 'EX' pad and inactivated by heat at 56°C for 1 hour). The medium is kept thoroughly mixed and then dispensed into sterile tubes. An advantage of this medium is that it is transparent, which facilitates the early detection of colonies.

iii) *Modified Middlebrook 7H10 (7)*

To prepare this medium 19 g Middlebrook 7H10 agar (Difco), 1g Casitone and 5 ml Glycerol are resuspended in 900 ml water, autoclaved at 121°C for 15 minutes and cooled to 58°C. Using an aseptic technique, the following additional ingredients are combined adding the egg yolk last: 50 ml PANTA PLUS (Becton Dickinson), 25 ml Mycobactin J solution (50 µg/ml), 100 ml ADC enrichment (Difco), 250 ml egg yolk. The mixture is thoroughly mixed using a slow swirling action and 10-ml volumes are dispensed into sterile tubes to form slopes. After a sterility check, media are stored at 4°C.

iv) *BACTEC 12B vials (7)*

The following supplements are added to each vial to give final concentrations of 0.8–1 µg/ml Mycobactin J and a minimum of 16–17% egg yolk in a final volume of 5–6 ml. For the 6-ml volume, 0.1 ml Mycobactin J (50 µg/ml), 0.1 ml PANTA PLUS, 1 ml egg yolk and 0.8 ml water are added. For the 5-ml volume, 0.1 ml Mycobactin J (50 µg/ml), 0.1 ml PANTA PLUS and 0.8 ml egg yolk are added.

v) *Middlebrook 7H9, 7H10 and 7H11 media (Difco)*

This media enhanced with mycobactin in the same proportion as for Herrold's medium can also be used. The advantage of this media is that it is transparent, which facilitates the early detection of colonies.

vi) *Löwenstein–Jensen medium with or without mycobactin (20).*

o **Sample preparation**

o *Processing tissue specimens*

Chemical preservatives should not be used. The tissues can be frozen at –20°C.

To avoid contamination, the faeces should be rinsed from portions of intestinal tract before shipment to the laboratory.

i) *Digestion/sedimentation method for decontamination of tissues*

Approximately 4 g of mucosa from the ileocaecal valve or 4 g of mesenteric node are placed in a sterile blender jar containing 50 ml of trypsin (2.5%). The mixture is adjusted to neutrality using 4% NaOH and pH paper, and stirred for 30 minutes at room temperature on a magnetic mixer. The digested mixture is filtered through gauze. The filtrate is centrifuged at approximately 2000–3000 g for 30 minutes. The supernatant fluid is poured off and discarded. The sediment is resuspended in 20 ml of 0.75% HPC and allowed to stand undisturbed for 18 hours at room temperature. The particles that settle to the bottom of the tube are to be used as the inoculum and are removed by pipette without disturbing the supernatant fluid. Alternatively, other methods of decontamination can be used, such as treatment with 5% oxalic acid.

ii) *Double incubation method for decontamination of tissues (7)*

About 2 g of tissue sample (trimmed of fat) is finely chopped using a sterile scalpel blade or scissors and homogenised in a stomacher for 1 minute in 25 ml 0.75% HPC. 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 the suspension from just above the sediment to a 30 ml tube and incubate for 3 hours at 37°C. Centrifuge for 30 minutes at 900 g, discard supernatant fluid and resuspend pellet in 1 ml antibiotic cocktail containing 100 µg of each of vancomycin, amphotericin and nalidixic acid (VAN). Incubate overnight at 37°C. Use the suspension to inoculate media as described below.

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and remove the egg white with the forceps and gravity. Make the hole larger and break the yolk. Mix the egg yolk by twirling the forceps, and remove the yolk sac. Pour the mixed egg yolk into media.

iii) *Inoculation of culture media and incubation*

Approximately 0.1 ml of inoculum is transferred to each of three slants of Herrold's medium containing mycobactin and to one slant of Herrold's medium without mycobactin. The inoculum is distributed evenly over the surface of the slants. The tubes are allowed to remain in a slanted position at 37°C for approximately 1 week with screw caps loose. The tubes are returned to a vertical position when the free moisture has evaporated from the slants. The lids are tightened and the tubes are placed in baskets in an incubator at 37°C.

The egg in Herrold's medium contributes sufficient phospholipids to neutralise the bactericidal activity of residual HPC in the inoculum. The other media (Modified Dubos and Middlebrook) do not have this property. Other treatments can be used for sample decontamination, for example oxalic acid at 5%.

HPC is relatively ineffective in controlling the growth of contaminating fungi. Amphotericin B (fungizone) was found to control effectively fungal overgrowth of inoculated media (31). Fungizone may be incorporated in the Herrold's medium at a final concentration of 50 µg per ml of medium. Due to loss of antifungal activity, storage of Herrold's medium containing fungizone should be limited to 1 month at 4°C.

The slants are incubated for at least 4 months and observed weekly from the sixth week onwards.

o *Processing faecal specimens*

No chemical preservative is used. The faecal specimens can be frozen at -70°C.

i) *Suspension and decontamination of faeces*

1 g of faeces is transferred to a 50 ml tube containing 20 ml of sterile distilled water. The mixture is shaken for 30 minutes at room temperature. The larger particles are allowed to settle for 30 minutes. The uppermost 5 ml of faeces suspension is transferred to a 50 ml tube containing 20 ml of HPC. The tube is inverted several times to assure uniform distribution and allowed to stand undisturbed for 18 hours at room temperature.

ii) *Inoculation of culture media*

0.1 ml of the undisturbed sediment is transferred to each of four slants of Herrold's medium, three with mycobactin and one without mycobactin. A smear may be made from the sediment and stained by the Ziehl-Neelsen method.

iii) *Incubation and observation of slants*

The same as for tissue specimens.

Variations in the above methods have been described (4, 24, 30, 35, 40, 54, 55). The sensitivity of culture may be enhanced using liquid media and with centrifugation rather than sedimentation techniques. The double incubation method described by Whitlock *et al.* (55) assists with decontamination of the inoculum (43) and offers higher sensitivity than the sedimentation or filtration protocols (11). The double incubation method involves mixing 2 g faeces with 15 ml saline or water followed by sedimentation for 30 minutes and transferring (avoiding fibrous matter) the top 5ml of the suspension to 25 ml of 0.9% HPC in half-strength brain-heart infusion (Difco). After incubating at 37°C for 16-24 hours, the mixture is centrifuged at 900 *g* for 30 minutes (room temperature), the supernatant is discarded and pellet resuspended in 1 ml VAN. The mixture is incubated for 24-72 hours at 37°C and used to inoculate media as described above (7).

d) **DNA probes and polymerase chain reaction**

DNA probes are being developed that offer a means of detecting *M. paratuberculosis* in diagnostic samples and of rapidly identifying bacterial isolates (12, 29). They have been used to distinguish between *M. paratuberculosis* and other mycobacteria.

McFadden *et al.* have identified a sequence (26, 27), termed IS900, which is an insertion sequence specific for *M. paratuberculosis*. It has been reported that a small number of isolates other than *M. paratuberculosis* have produced amplified products the same size as expected from *M. paratuberculosis*. A restriction enzyme digest may be applied to positive IS900 products to confirm that their sequence is consistent with *M. paratuberculosis* (6).

The identifications of new DNA sequences considered to be unique to *M. paratuberculosis*; ISMav2, f57, and ISMap02 sequences, offer additional tools for rapid identification of this organism using the polymerase chain reaction (PCR) technology (44, 45, 51). The restriction enzyme analysis of IS1311, an insertion sequence common to *M. avium* and *M. paratuberculosis* can be used to distinguish between these species and for typing of ovine, bovine and bison strains of *M. paratuberculosis* (38, 57).

The use of IS900 as a DNA probe for specific identification of *M. paratuberculosis* in faecal samples from cattle by PCR has been reported (52). Commercial diagnostic PCR tests for the detection of *M. paratuberculosis* in milk and faecal samples have been developed.

## 2. Serological tests

The serological tests commonly used for paratuberculosis in cattle are complement fixation (CF), enzyme-linked immunosorbent assay (ELISA) and agar gel immunodiffusion (AGID) (42) corresponding to humoral immunity, and the gamma interferon assay corresponding to cellular immunity.

### a) Complement fixation test

The CF test has been the standard test used for cattle for many years. The CF test works well on clinically suspect animals, but does not have sufficient specificity to enable its use in the general population for control purposes. Nevertheless, it is often demanded by countries that import cattle. A variety of CF test procedures are used internationally. An example of a microtitre method for performing the CF test is as follows:

- i) The antigen is an aqueous extract of bacteria from which lipid has been removed (strain *M. paratuberculosis* 316F). *Mycobacterium avium* D9 may also be used.
- ii) All sera are inactivated in the water bath at 60°C for 30 minutes and diluted at 1/4, 1/8 and 1/16. A positive control serum and a negative control serum should be included on each plate. The following controls are also prepared: antigen control, complement control and haemolytic system control.
- iii) Reconstituted, freeze-dried complement is diluted to contain six times H<sub>50</sub> (50% haemolysing dose) as calculated by titration against the antigen.
- iv) Sheep erythrocytes, 2.5%, are sensitised with 2 units of H<sub>100</sub> haemolysin.
- v) All dilutions and reagents are prepared in calcium/magnesium veronal buffer; 25 µl volumes of each reagent are used in 96-well round-bottom microtitration plates.
- vi) Primary incubation is at 4°C overnight and secondary incubation is at 37°C for 30 minutes.
- vii) *Reading and interpreting the results:* Plates may be left to settle or centrifuged and read as follows: 4+ = 100% fixation, 3+ = 75% fixation, 2+ = 50% fixation, 1+ = 25% fixation and 0 = complete haemolysis. The titre of test sera is given as the reciprocal of the highest dilution of serum giving 50% fixation. A reaction of 2+ at 1/8 is regarded as positive. Results should be interpreted in relation to clinical signs and other laboratory findings.

### b) Enzyme-linked immunosorbent assay

The ELISA is, at present, the most sensitive and specific test for serum antibodies to *M. paratuberculosis* in cattle. Its sensitivity is comparable with that of the CF test in clinical cases, but is greater than that of the CF test in subclinically infected carriers. The specificity of the ELISA is increased by *M. phlei* absorption of sera. The absorbed ELISA, designed by Yokomizo *et al.* (61, 62) and modified by Milner *et al.* (32), was developed into a commercial kit by Cox *et al.* (8).

The ELISA detects about 30–40% cattle identified as infected by culture of faeces on solid media (56). Similarly to the culture methods, the sensitivity of the ELISA depends on the level of *M. paratuberculosis* shedding in faeces and the age of animals. A large study recently performed in Australia showed that 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 (22). The overall actual sensitivity for all age-groups was calculated to be about 15% (22, 56).

In small ruminants the commercially available ELISA had a specificity of 98.2–99.5% (95% confidence intervals [CI]) and detected 35–54% (95% CI) of animals with histological evidence of infection (18). In another study the estimated specificity of an in-house ELISA was 99% and its sensitivity measured against histological results was 21.9% (37).

The absorbed ELISA combines the sensitivity of ELISA with the added specificity of an absorption step. Sera to be tested are diluted with buffer containing soluble *M. phlei* antigen prior to testing in an indirect ELISA. This procedure eliminates nonspecific cross-reacting antibodies. In early versions, sera were absorbed with whole *M. phlei*, which were removed by centrifugation prior to testing.

A microtitre plate format has been developed in which *M. paratuberculosis* antigen is coated on to 96-well plates. Samples are diluted in sample diluent containing *M. phlei* to remove cross-reacting antibodies. On incubation of the diluted sample in the coated well, antibody specific to *M. paratuberculosis* forms a complex with the coated antigens. After washing away unbound materials from the wells, horseradish peroxidase (HPRO)-labelled anti-bovine immunoglobulin is added. This reacts with immunoglobulins bound to the solid-

phase antigen. The rate of conversion of substrate is proportional to the amount of bound immunoglobulin. Subsequent colour, measured (at 450 nm) spectrophotometrically is proportional to the amount of antibody present in the test sample.

The antigen used to coat the ELISA plates is available commercially.

An anti-bovine IgG labelled with HRPO is used as conjugate. The substrate chromogen solution is hydrogen peroxide tetramethyl benzidine. A solution of 0.5 M H<sub>2</sub>SO<sub>4</sub> is used to stop the reaction when the absorbance of the positive control serum reaches a predetermined point.

Several absorbed ELISA kits are commercially available. The method and test materials needed, the interpretation of the results and calculations are fully described in the instructions accompanying the commercial kit. It has recently been reported that several commercially available ELISAs have similar sensitivities and specificities (5). Some commercial kits offer an option of testing milk samples. The ELISA on bovine and caprine milk has been found to have specificity similar to that of the serum ELISA, but less sensitive than the blood test (17, 36).

### c) Agar gel immunodiffusion test

The AGID test is useful for the confirmation of the disease in clinically suspect cattle, sheep and goats (39). 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 ELISAs (15, 18, 37). The reported specificity and sensitivity of the AGID measured against histological results were 99–100% (95%CI) and 38–56% (95% CI), respectively (18).

The antigen employed is a crude protoplasmic extract of laboratory strain *M. avium* 18 (formally *M. paratuberculosis* 18) prepared by disruption of cells in a hydraulic press cell fractionator. Disrupted cells are centrifuged at 40,000 *g* for 2 hours to remove cell wall debris, and the supernatant fraction is retained and lyophilised. This antigen is resuspended in water at a concentration of 10 mg/ml.

Agarose is dissolved in barbital buffer, pH 8.6, containing sodium azide, to give a final agarose concentration of 0.75%. Agarose may be poured into Petri dishes or on to glass slides. Wells are cut in a hexagonal pattern. Wells are 4 mm in diameter, 4 mm apart, and the agar should be 3–4 mm deep. Antigen is added to centre wells. Test, positive and negative control sera are added to alternate peripheral wells.

Plates are incubated in a humid chamber at room temperature. Gels are examined for precipitation lines after 24 and 48 hours' incubation. The appearance of one or more clearly definable precipitation line(s), showing identity with that of a control positive serum, before or at 48 hours, constitutes a positive test result. Absence of any precipitation lines is recorded as a negative test result. Nonspecific lines may occur.

Several variations of the method are in use.

## 3. Tests for cell-mediated immunity

The detection of a systemic cell-mediated response precedes detectable antibody production. Animals that are minimally infected frequently fail to react on serological testing but may react positively to tests that measure cell-mediated immunity.

### a) Gamma interferon assay

The assay is based on the release of gamma interferon from sensitised lymphocytes during an 18–36-hour incubation period with specific antigen (avian purified protein derivative [PPD] tuberculin, bovine PPD tuberculin or johnin) (60). The quantitative detection of bovine gamma interferon is carried out with a sandwich ELISA that uses two monoclonal antibodies to bovine gamma interferon. A commercial diagnostic test based on the detection of gamma interferon has been developed for the diagnosis of bovine tuberculosis. The method and test materials needed 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 paratuberculosis. 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. In cattle the reported specificity of the test varied from 94% to 67% depending on the interpretation criteria (23).

### b) Delayed-type hypersensitivity

The skin test for delayed-type hypersensitivity (DTH) is a measure of cell-mediated immunity, but has limited value. The test is carried out by the intradermal inoculation of 0.1 ml of antigen into a clipped or shaven site, usually on the side of the middle third of the neck. In the past, avian PPD tuberculin<sup>3</sup> or johnin was used for this purpose as it was believed that avian tuberculin and johnin are of comparable sensitivity and specificity. The skin thickness is measured with calipers before and 72 hours after inoculation. Increases in skin thickness of over 2 mm should be regarded as indicating the presence of DTH. 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 (19). Furthermore the interpretation of the skin test results is complicated by the lack of agreement with respect to interpretation criteria. In a recent study in which johnin (ID-Lelystad, Lelystad, The Netherlands) was used to test cattle, the skin test specificity was 88.8% at the cut-off value of  $\geq 2$  mm, 91.3% at the cut-off value of  $\geq 3$  mm and 93.5% at the cut-off value of  $\geq 4$  mm (23). 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 (23). Further research is required to increase the value of the skin test.

### C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

**Vaccines:** Vaccines used against paratuberculosis are: live, attenuated, incorporated with oil and pumice; lyophilised, live, attenuated, which may be adjuvanted with, for example, oil after reconstitution; and heat-killed bacterins. Vaccines may be prepared from one strain of *M. paratuberculosis* 316F or 2E (Weybridge) or *M. paratuberculosis* 3 and 5 or II (Canadian strains), or as many as three strains may be used. The information below applies to a live, attenuated vaccine adjuvanted with oil and pumice (10, 50, 59). Vaccination may cause a reaction at the site of injection. Vaccination may also interfere with eradication programmes based on immunological testing and elimination of animals identified as infected and can interfere with the interpretation of DTH skin tests for bovine tuberculosis.

**Diagnostic products:** Johnin PPD is a preparation of the heat-treated products of growth and lysis of *M. paratuberculosis*. Avian tuberculin PPD is a preparation of heat-treated products of growth and lysis of *M. avium* D4ER or TB 56. Details of avian tuberculin PPD are in Chapter 2.3.6 Avian tuberculosis. These two preparations are used, by intradermal injection, to reveal DTH as a means of identifying animals infected or sensitised with *M. paratuberculosis*.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8. Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

#### 1. Seed management

##### a) Characteristics of the seed

**Vaccine:** Seed strains should be of a prevalent type, which may be checked by biotyping or genetic analysis. They should have been demonstrated to be innocuous when administered by the recommended route of vaccination to intended target species.

**Johnin:** Strains of *M. paratuberculosis* used to prepare seed cultures should be identified by biotyping or genetic tests. They should be shown to be free from contaminating organisms.

##### b) Method of culture

**Vaccine:** Seed cultures may be made on potato slants partly immersed in a suitable medium, such as Reid's synthetic medium<sup>4</sup> (53). Cultures may be stored lyophilised. Active cultures are normally incubated at 37°C.

3 Avian tuberculin can be obtained from VLA Weybridge, New Haw, Addlestone, Surrey KT15 3NB, United Kingdom, or from Symbiotic Society, 299 av. Jean Jaurès, 69007 Lyon, France.

4 L-Asparagine, 5.0 g  
Potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ , anhydrous), 2.0 g  
Magnesium sulphate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ), 1.0 g  
Ammonium citrate ( $[\text{NH}_4]_3 \text{C}_6\text{H}_5\text{O}_7$ ), 2.0 g  
Sodium chloride, 2.0 g  
Ferric ammonium citrate, 0.075 g  
Dextrose monohydrate B.P., 10 g  
Glycerol B.P. (48 ml), 60 g  
Distilled water to 1000 ml

*Johnin*: The culture substrate should be shown to be capable of producing a product free from substances known to cause toxic or allergic reactions. A suitable medium for seed culture is that of Reid, solidified with 1.75% agar, in screw cap tubes. Cultures may also be stored lyophilised.

**c) Validation as a vaccine**

*Vaccine*: Purity tests should be carried out on seed cultures and final harvest by stained smears.

The vaccine should be used as part of a control programme and will not on its own provide complete protection against disease caused by *M. paratuberculosis* (59). There is usually good control of clinical disease, but subclinical infection persists in vaccinated herds, albeit at a reduced level. Vaccine should be administered to animals in early life only, e.g. calves in their first month of life. It should be inoculated subcutaneously and causes a small inflammatory swelling. This is gradually replaced by a cold, painless, fibro-caseous nodule, which varies in size and which may persist for years. Vaccination has been used to control the disease in sheep and goats, including older animals. In order to get the best results from vaccination, management practices to control the disease should also be in place.

The use of vaccines may interfere with the outcome of diagnostic skin tests for tuberculosis, and this should be remembered when planning a control programme (16).

*Johnin*: Cultures should be checked by staining smears for the presence of contaminating organisms.

To test for lack of sensitising effect, three guinea-pigs that have not previously been treated with any material that could interfere with the test, are each injected intradermally on each of three occasions at 5-day intervals, with 0.01 mg of the preparation under test in a volume of 0.1 ml. Each guinea-pig, together with each of three control guinea-pigs that have not been injected previously, is injected intradermally 15–21 days after the third injection with the same dose of the same johnin. The reactions of the two groups of guinea-pigs should not be significantly different when measured 24–48 hours later.

**2. Method of manufacture**

*Vaccine*: For vaccine batches, the organisms may be grown on a liquid synthetic medium, such as Reid's synthetic medium. The organisms grow as a pellicle on the liquid surface. To ensure a good surface area, it is convenient to use vessels such as conical flasks containing one-third of their nominal volume of liquid medium. These flasks may be seeded directly from potato slant cultures, but with some strains, one or more passages on liquid medium may be necessary to ensure adequate pellicle growth for the final, vaccine batch passage. Such passaging should usually take place at 2-week intervals as longer periods may result in over-maturation and sinking of the pellicle. Incubation is at 37°C.

To prepare the vaccine, the pellicle growth from 2-week-old cultures of each strain to be included may be separated from the liquid medium by decantation, filtration and pressing between filter paper pads. The moist *M. paratuberculosis* culture is blended with an adjuvant, such as liquid paraffin, olive oil and pumice (10).

*Johnin*: Johnin for skin test diagnosis is a PPD prepared from one or more strains of *M. paratuberculosis* (available from VLA Weybridge or CDI, Lelystad, the Netherlands). It may be prepared by the following method.

*Mycobacterium paratuberculosis* strains are grown as a pellicle on liquid Reid's medium. Production cultures are usually inoculated from liquid seeding cultures rather than directly from seed on solid medium (Reid's synthetic medium). Production cultures are incubated at 37°C for 10 weeks.

At the end of the incubation period, the culture medium has a pH of about 5 and little or no johnin will be obtained unless the pH is raised, using sodium hydroxide, to about 7.3 before steaming. After thorough mixing, the cultures are free steamed for 3 hours. The bulk of the killed organisms is removed by coarse filtration and the filtrate is clarified by further filtration. Protein in the filtrate is precipitated chemically with 40% trichloroacetic acid, washed and redissolved (alkaline solvent). The product is sterilised by filtration. An antimicrobial preservative that does not give rise to false-positive reactions, such as phenol (not more than 0.5% [w/v]), may be added. Glycerol (not more than 10% [w/v]) may be added as a stabiliser. The product is dispensed aseptically into sterile glass containers, which are then sealed.

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pH (not adjusted) 5.6–5.8

When required, the above medium is solidified by the addition of 1.5% granulated agar (Difco). Sterilised at 121°C for 15 minutes.

### 3. In-process control

*Vaccine:* Adequate growth of culture and cultural purity need to be checked. Presence of contaminating organisms may be detected by conventional sterility tests on harvests. Tests for pathogenic mycobacteria are carried out by injection of moist culture, taken prior to blending with adjuvant and diluted tenfold in saline, into two guinea-pigs, each receiving 1 ml. These are observed for 8 weeks, killed humanely, and examined for any abnormal lesions.

*Johnin:* After final filtration the sterility of each filtrate of the PPD solution is checked.

Sterile filtrates are tested for protein content by a Kjeldahl method (1). The protein content is adjusted to give between 0.475 and 0.525 mg/ml of protein in the final product. The pH is adjusted to the range 6.5–7.5.

### 4. Batch control

#### a) Sterility

Tests for sterility and freedom from contamination may be found in Chapter 1.1.9 Tests for sterility and freedom from contamination of biological materials. The vaccine organism will not normally grow to a detectable level in conventional sterility tests.

#### b) Safety

*Vaccine:* These tests are normally performed in laboratory animals, although multidose tests in target animals would also be satisfactory. A typical laboratory animal test would be as follows. Each of two guinea-pigs is inoculated, subcutaneously, with an acceptable batch of vaccine at a fraction of the cattle dose previously determined to give a nodule but no overt necrosis at the injection site. Animals are observed for 8 weeks, killed humanely and examined for any abnormal lesions.

*Johnin:* Two guinea-pigs should each be injected subcutaneously with 0.5 ml of the johnin under test. No significant local or systemic lesions should be seen within 7 days (1).

Tests on johnin for living mycobacteria may be performed either on the material immediately before it is dispensed into final containers or on samples taken from final containers themselves. A sample of at least 10 ml should be taken, and this should be injected intraperitoneally or subcutaneously into at least two guinea-pigs, dividing the volume to be tested equally between the guinea-pigs. It is desirable to take a larger sample, say 50 ml, and to concentrate any residual mycobacteria by centrifugation or membrane filtration. The guinea-pigs are observed for at least 42 days and post-mortem examinations are carried out. Any macroscopic lesions are examined microscopically and culturally.

#### c) Potency

*Vaccine:* As protection tests appear to be impractical, a test of sensitising ability may be used. This may then be related to bacterial content based on weight. A typical test would be as follows: guinea-pigs are sensitised by intramuscular injection of 0.5 ml of a 100-fold dilution in liquid paraffin of the vaccine under test. Skin tests are performed 6 weeks after sensitisation using intradermal inoculations of 0.2 ml of at least three serial dilutions of an *M. paratuberculosis* antigen, such as johnin PPD, the dilutions being chosen to give expected skin reactions of from 8 mm to 25 mm diameter. Each guinea-pig receives several dilutions per flank, their distribution being chosen by a Latin square design. After 24–48 hours, skin reactions are measured. A reference preparation for tests of this type has not yet been fully established. Avian tuberculin PPD of known international unitage may be used as a skin test antigen in tests of this type to ensure that the vaccine is capable of producing adequate sensitisation (corresponding to the vaccination).

*Johnin:* The potency of johnin is currently determined by chemical assay for protein using a Kjeldahl method. A PPD content of  $0.5 \pm 0.025$  mg/ml of final product is recommended (1).

The identity of the material should be confirmed by injecting intradermally into guinea-pigs sensitised by injections of killed *M. paratuberculosis* (100 mg powder mycobacteria + 25 ml vaseline + 100 mg pumice stone) 6 weeks previously.

It is possible to perform a potency test using dilutions of johnin in guinea-pigs sensitised with *M. paratuberculosis*, similar to such tests for the potency of bovine and avian tuberculin, but a standard preparation for this type of test has not yet been fully established.

#### d) Duration of immunity

*Vaccine:* After vaccination at the age of 14–30 days, the vaccination effect is expressed as the reduction in the rate of excretors among vaccinated animals as compared with nonvaccinated bovines (21).

There is usually good control of clinical disease, but a reduced level of subclinical infection persists. The favourable results probably reflect a diminishing exposure to infection resulting from a reduction in the number of heavy excretors in the herd.

**e) Stability**

*Vaccine:* The vaccine may be stored at 2–8°C for 9–12 months without loss of potency. It should not be frozen.

*Johnin:* Johnin should be protected from light and stored at 2–8°C. Under these conditions it should retain its potency for at least 5 years.

**f) Preservatives**

A preservative is normally included for vaccine in multidose containers. For johnin, the phenol used is no more than 0.5% (w/v). The concentration of the preservative in the final product and its persistence through shelf life should be checked.

**g) Precautions (hazards)**

*Vaccine:* The vaccine causes some side-effects, nodule formation and sensitisation of animals to the tuberculin test (15). In humans, accidental injection of vaccine has resulted in chronic inflammatory reactions requiring surgical treatment (21).

**5. Tests on the final product**

**a) Safety**

See Section C.4.b.

**b) Potency**

See Section C.4.c.

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**NB:** There are OIE Reference Laboratories for Paratuberculosis (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: [www.oie.int](http://www.oie.int)).