CHAPTER 3.1.16.

PARATUBERCULOSIS
(JOHNE'S DISEASE)

SUMMARY

Description of the disease: *Paratuberculosis (Johne's disease) is a chronic enteritis of ruminants caused by* Mycobacterium avium *subsp.* paratuberculosis *(MAP).*

Diagnosis of paratuberculosis is made on clinical grounds confirmed by the demonstration of MAP *in the faeces by microscopy, culture, or by the use of DNA probes and the polymerase chain reaction (PCR). 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* MAP *in culture.*

Detection 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.*

The detection of subclinical infection depends on the detection of specific antibodies by serology, culture of MAP *from faeces or tissues collected at necropsy, PCR, or the demonstration of cell-mediated responses. The choice of test depends on the circumstances and the degree of sensitivity and specificity required at individual animal or herd level.*

Cultures of MAP *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* MAP.

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 test commonly used for paratuberculosis in cattle is absorbed enzyme-linked immunosorbent assay (ELISA). Agar gel immunodiffusion remains a valuable test for the detection of paratuberculosis in sheep. 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 ELISA performs very well.

Tests for cell-mediated immunity: *Interferon-gamma release assay and delayed type hypersensitivity methods (skin test) have been used, but interpretation in both cases is difficult.*

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 MAP *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* MAP. *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* (*MAP*) is an organism first observed by Johne & Frothingham in 1895. *MAP* causes paratuberculosis or Johne's disease, an intestinal granulomatous infection (Thorel *et al*., 1990). Paratuberculosis is found most often among domestic ruminants (cattle, sheep, goats, camelids and buffaloes) as well as wild ruminants (cervids) and has a global distribution. The disease has also been reported in horses, donkeys, pigs, rabbits, stoats, foxes and weasels (Greig *et al*., 1999). Under natural conditions, the disease in cattle spreads by ingestion of *MAP* from the contaminated environment. The disease persists after the introduction of infected animals. Infection can be spread vertically to the fetus (Larson & Kopecky, 1970) and semen can be infected with the organism (Sweeney *et al*., 1995). The primary source of infection in calves is faeces from infected cows or milk that is contaminated with the faeces of diseased cattle. Isolates from sheep have different characteristics from those in cattle, but the laboratory detection and diagnostic methods are the same.

The identification of *MAP* is based on its mycobactin requirement and on its association with clinical signs and defined laboratory findings, such as culture and PCR results. Mycobactin dependence has long been used as a taxonomic characteristic for *MAP* because most mycobacteria are able to make mycobactin for themselves. *MAP, 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 *MAP;* this characteristic exists to various degrees within the *M. avium* group.

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. It is also reported as a significant clinical sign in farmed deer. 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. *MAP* 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 muscle wasting. Clinical signs usually first appear in young adulthood, but the disease can occur in animals at any age over 1–2 years and in dairy cattle is most frequently reported in the 3- to 5-year old age group.

Within a few weeks of infection, a phase of multiplication of *MAP* begins in the walls of the small intestine. Depending on the natural 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 *MAP* in the faeces. In most cases larger numbers of organisms are excreted as clinical disease develops.

Cell-mediated immune responses (CMI) are detectable early in the infection and remain present in a proportion of the subclinically infected carriers, but as the disease progresses, CMI wanes and may be absent in clinical cases. Serum antibodies are detectable later than CMI. They may also be present in animals 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, stimulate CMI 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 CMI reactions.

Animals vaccinated against paratuberculosis with whole cell vaccines develop both CMI 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, it is advisable to use tests detecting the antigen *MAP* in samples of faeces or tissues.

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 *MAP*.

MAP organisms and potentially infected specimens should be handled at an appropriate biosafety and containment level determined by biorisk analysis as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for diagnosis of paratuberculosis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent[a]						
Histopathology*	+	–	+	+++	–	–
Faecal ZN staining	–	–	–	+	–	–
Culture	–	+	+	+++	+	–
PCR	+++	+++	+	++	+	–
Detection of immune response[b]						
AGID**	++	+	+	++	+++	+++
ELISA	+++	+++	+++	+	+++	+++
IFN-γ release assay	–	–	+	–	–	+++
DTH	–	–	+	–	–	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose;
* = post-mortem use only; ** = appropriate for the use in sheep and goats.
ZN = Ziehl–Neelsen; PCR = polymerase chain reaction; AGID = agar gel immunofiffusion; ELISA = enzyme-linked immunosorbent assay; IFN-γ = gamma interferon; DTH = delayed-type hypersensitivity.
[a]A combination of agent identification methods applied on the same clinical sample is recommended.
[b]A combination of the listed serological tests is recommended.

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 (Table 1).

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. Similar test strategies with repeated tests at the herd level can also be applied within control programmes to estimate herd-level probabilities of freedom from infection, and thus to identify low risk herds for safer trade.

1. Detection of the agent

1.1. 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 lymphatics. The mesenteric lymph nodes are usually enlarged and oedematous. Caseous and/or calcified lesions in mesenteric lymph nodes are often seen in goats and to a lesser extent in sheep (Fodstad & Gunnarsson, 1979). Smears from the affected mucosa and cut surfaces of lymph nodes can be stained by Ziehl–Neelsen's method and examined microscopically for acid-fast organisms that have the morphological characteristics of *MAP*. 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.

1.2. 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.

1.3. Bacteriology (culture)

The isolation of *MAP* 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). However, due to the potential pass-through phenomenon, it is theoretically possible that faecal culture testing of non-infected animals on contaminated premises can lead to false-positive reactions (Nielsen & Toft, 2008).

The faecal culture is widely considered to be the gold standard for the diagnosis of paratuberculosis in live animals (affected animals). Actually, 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 (Nielsen & Toft, 2008) according to the conditions, sensitivity of faecal culture is 70% for affected cattle, 74% for infectious cattle and 23–29% for infected cattle. The culture of bovine, ovine and caprine tissues for *MAP* is more sensitive than histopathological examination (Fodstad & Gunnarsson, 1979; Koh *et al.,* 1988; Whittington *et al.,* 1999).

There are several culture methods, which vary with respect to media and sample processing protocols. The cultivation of *MAP* is always performed using special media supplemented with mycobactin J, which is available commercially.

MAP organisms are vastly outnumbered by other bacteria or fungi in faecal and intestinal tissue specimens. The successful isolation of *MAP* 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 *MAP*. Routine decontamination protocols were shown to decrease the number of *MAP* organisms isolated per sample by about 2.7 $\log_{10}$ and 3.1 $\log_{10}$ for faeces and tissues, respectively.

There are two basic methods of sample decontamination: 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 solid media such as

Herrolds's egg yolk medium (HEYM) or Middlebrook 7H10 and liquid media such as Middlebrook 7H9 or commercial equivalents for growth. Although it has been published that HEYM supports growth of bovine isolates of *MAP* significantly better than LJ, recent studies have shown that certain strains grow better on LJ or Middlebrook media. The advantage of 7H10 medium is that it better supports the growth of ovine strains compared with HEYM. Liquid media have been reported to be more sensitive than solid media for both ovine and bovine strains.

Primary colonies of *MAP* on solid media may be expected to appear any time from 5 weeks to 6 months after inoculation. 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 supplemented with egg yolk and mycobactin is excellent for the cultivation of ovine strains of *MAP* (Whittington *et al*., 1999).

Primary colonies of the cattle strain of *MAP* 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.

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

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

For identification of *MAP,* 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 *MAP* on medium that contains no mycobactin. In addition, the PCR confirmation (targeting IS*900* or F57) should be used to confirm the identification of *MAP* isolates.

1.3.1. Media

Examples of suitable media are:

i)   Herrold's egg yolk medium with mycobactin

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[1] and sterile malachite green solution. Blend gently and dispense into sterile tubes.

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

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1   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, 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.

ii)   Modified Dubos's medium

For 1 litre of medium: 2.5 g 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 and 7H9

Middlebrook media with added Mycobactin and various commercially available supplements can be used. Further advice on formulation can be obtained from the WOAH Reference Laboratories.

iv)   Löwenstein–Jensen medium with or without mycobactin

1.3.2. Sample preparation

i)   Processing tissue specimens

Chemical preservatives should not be used. The tissues can be frozen at –70°C.

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

a)   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.

b)   Double incubation method for decontamination of tissues

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.

c)   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. 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.

ii) Processing faecal specimens

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

a) 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 0.95% HPC. The tube is inverted several times to assure uniform distribution and allowed to stand undisturbed for 18 hours at room temperature.

b) 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.

c) Incubation and observation of slants

The same as for tissue specimens.

Variations in the above methods have been described (Collins *et al.*, 1990; Merkal *et al.*, 1968). 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.* (1991) assists with decontamination of the inoculum and offers higher sensitivity than the sedimentation or filtration protocols (Eamens *et al.*, 2000). 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 5 ml of the suspension to 25 ml of 0.9% HPC in half-strength brain–heart infusion. 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.

1.4. DNA probes and polymerase chain reaction

DNA probes are being developed that offer a means of detecting *MAP* in diagnostic samples and of rapidly identifying bacterial isolates (Ellingson *et al.*, 1998). They have been used to distinguish between *MAP* and other mycobacteria.

McFadden *et al.* have identified a sequence (McFadden *et al.*, 1987), termed IS*900*, which is an insertion sequence specific for *MAP* (Vary *et al.,* 1990). It has been reported that a small number of isolates other than *MAP* have produced amplified products the same size as expected from *MAP*. A restriction enzyme digest may be applied to positive IS*900* products to confirm that their sequence is consistent with *MAP*.

The identifications of new DNA sequences considered to be unique to *MAP* (ISMav2, f57, and ISMap02 sequences), offer additional tools for rapid identification of this organism using the polymerase chain reaction (PCR) technology (Stabel & Bannantine, 2005; Strommenger *et al.*, 2001; Vansnick *et al.*, 2004). The restriction enzyme analysis of IS*1311*, an insertion sequence common to *M. avium* subsp. *avium* and

MAP can be used to distinguish between these species and for typing of ovine, bovine and bison strains of *MAP* (Sevilla *et al*., 2005; Whittington *et al*., 1998).

In recent years, real-time PCR methods have been extensively developed to detect *MAP* from different specimens (blood, milk, faeces, tissues and environmental samples). The technique is rapid and offers hope for detection of fastidious and slow growing microorganisms, such as *MAP*. However, this molecular tool is greatly influenced by the quality of nucleic acid samples. Therefore, a DNA extraction method that provides a high quality DNA sample and a maximum bacterial DNA recovery is a critical step to use real-rime PCR (Parka *et al*., 2014).

PCR assays have now improved to the point where they play an important role in the diagnosis of disease (Leite *et al.,* 2013).

Commercial diagnostic PCR tests for the detection of *MAP* in milk and faecal samples are available but users should consider the interpretation of acquired data and fitness for purpose before adopting such methods.

2. Serological tests

The serological test commonly used for paratuberculosis in cattle is enzyme-linked immunosorbent assay (ELISA). The complement fixation test (CFT) and agar gel immunodiffusion (AGID) show poorer sensitivity and specificity and are no longer recommended in cattle. However AGID remains a valuable test for the detection of paratuberculosis in sheep. There is no international reference serum.

2.1. Enzyme-linked immunosorbent assay

The ELISA is, at present, the most sensitive and specific test for serum antibodies to *MAP* in cattle. Its sensitivity 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. The absorbed ELISA, designed by Yokomizo *et al*. (1983; 1985) and modified by Milner *et al*. (1988), was developed into a commercial kit by Cox *et al*. (1991).

The ELISA detects about 30–40% cattle identified as infected by culture of faeces on solid media (Whitlock *et al*., 2000). Similarly to the culture methods, the sensitivity of the ELISA depends on the level of *MAP* shedding in faeces and the age of animals. A large study 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 (Jubb *et al*., 2004). The overall actual sensitivity for all age-groups was calculated to be about 15% (Jubb *et al*., 2004; Whitlock *et al*., 2000). In cattle, the sensitivities of ELISA are in the range 7–94%, and the specificities of ELISA are in the range 40–100% (Nielsen & Toft, 2008).

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 (Hope *et al*., 2000). In another study, the estimated specificity of an in-house ELISA was 99% and its sensitivity measured against histological results was 21.9% (Sergeant *et al*., 2003). In small ruminants, the sensitivities of ELISA are in the range 16–100%, and the specificities of ELISA are in the range 79–100% (Nielsen & Toft, 2008).

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 *MAP* 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 *MAP* forms a complex with the coated antigens. After washing away unbound materials from the wells, horseradish peroxidase (HRPO)-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 spectrophotometrically (at the wavelength appropriate to the chromogen used) is proportional to the amount of antibody present in the test sample.

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 been reported that several commercially available ELISAs have similar sensitivities and specificities. 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 (Hendrick *et al*., 2005; Salgado *et al*., 2005). In cattle, the sensitivities of milk ELISA are in the range 21–61%, and the specificities of milk ELISA are in the range 83–100% (Nielsen & Toft, 2008).

2.2. Complement fixation test

The CFT has been the standard test used for cattle for many years. The CFT works well on clinically suspect animals, but does not have sufficient specificity to enable its use in the general population for control purposes. Thus, the CFT is not recommended for control purposes nor for individual animal testing prior to international movement. A variety of CFT procedures are used internationally. There are no international pattern sera with standardised complement fixation units for use as a reference. An example of a microtitre method for performing the CFT is given.

2.1.1. Test procedure

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_{50} (50% haemolysing dose) as calculated by titration against the antigen.

iv)   Sheep erythrocytes, 2.5%, are sensitised with 2 units of H_{100} 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.

2.3. Agar gel immunodiffusion test

The AGID test is useful for the confirmation of the disease in clinically suspect cattle, sheep and goats. 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 (Gwozdz *et al*., 2000; Hope *et al*., 2000; Sergeant *et al*., 2003). The reported specificity and sensitivity of the AGID measured against histological results were 99–100% (95%CI) and 38–56% (95% CI), respectively (Hope *et al*., 2000).

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 CMI. In infected populations, a much higher number of animals is expected to react in tests for CMI compared with antibody tests, as CMI is indicative of exposure while antibodies indicate progress of infection.

3.1. Interferon-gamma release assay

The assay is based on the release of gamma interferon (IFN-γ) from sensitised lymphocytes during an 18–36-hour incubation period with specific antigen (avian purified protein derivative [PPD] tuberculin, bovine PPD tuberculin or johnin[2]). The quantitative detection of bovine IFN-γ is carried out with a sandwich ELISA that uses two monoclonal antibodies to bovine IFN-γ. A commercial diagnostic test based on the detection of IFN-γ 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 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% and the sensitivity varied from 13% to 85%, depending on the interpretation criteria (Kalis *et al.*, 2003; Nielsen &Toft, 2008).

Several ELISA kits are commercially available for quantitative detection of IFN-γ on bovine, ovine and caprine plasmas.

3.2. 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 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. Furthermore the interpretation of the skin test results is complicated by the lack of agreement with respect to interpretation criteria. In a study in which johnin 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 (Kalis *et al.*, 2003). 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 (Kalis *et al.*, 2003). Further research is required to increase the value of the skin test.

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2   Johnin can be obtained from ID-Lelystad, The Netherlands

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

C1. Vaccines

1. Background

Vaccination may 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. All commercial vaccines now available are whole-cell-based vaccines. Vaccination of cattle or small ruminants is not allowed in many countries mainly due to the fear of giving rise to false positive results in the skin test for bovine tuberculosis.

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.

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

2.1.1. Quality criteria

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

2.1.2. Validation as a vaccine strain

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.

2.2. Method of manufacture

2.2.1. Procedure

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 *MAP* culture is blended with an adjuvant, such as liquid paraffin, olive oil and pumice.

2.2.2. In-process controls

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.

2.2.3. Final product batch test

i) Sterility

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

ii) Identity

Identity of culture is performed by Ziehl–Neelsen staining and PCR (see Section B.1.4).

iii) Safety

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.

iv) Batch potency

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 *MAP* 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).

2.3. Requirements for regulatory approval

2.3.1. Manufacturing process

A preservative is normally included for vaccine in multidose containers.

2.3.2. Safety requirements

i) Target and non-target animal safety

See Section C1.2.2.3.

ii) Reversion-to-virulence for attenuated/live vaccines and environmental considerations

Live vaccines are not available.

iii) Precautions (hazards)

The vaccine causes some side-effects, nodule formation and sensitisation of animals to the tuberculin test (Gwozdz *et al.*, 2000). In humans, accidental injection of vaccine has resulted in chronic inflammatory reactions requiring surgical treatment.

2.3.3. Efficacy requirements

The vaccine should be used as part of a control programme and will not on its own provide complete protection against disease caused by *MAP*.

2.3.4. Vaccines permitting a DIVA strategy (detection of infection in vaccinated animals)

No DIVA vaccine yet available

2.3.5. Duration of immunity

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.

C2. Johnin

1.   Background

Johnin PPD is a preparation of the heat-treated products of growth and lysis of *MAP*. Avian tuberculin PPD is a preparation of heat-treated products of growth and lysis of *M. avium* subsp. *avium* D4ER or TB 56. Details of avian tuberculin PPD are in Chapter 3.3.6 *Avian tuberculosis*. These two preparations are used, by intradermal injection, to reveal DTH as a means of identifying animals infected or sensitised with *MAP.*

Guidelines for the production of veterinary biologicals 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.

2.   Outline of production and minimum requirements for Johnin

2.1.   Characteristics of the seed

2.1.1.   Quality criteria

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.

2.1.2.   Validation as a strain

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

2.2.   Method of manufacture

2.2.1.   Procedure

Johnin for skin test diagnosis is a PPD prepared from one or more strains of *MAP*. It may be prepared by the following method.

MAP 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.

2.2.2. In-process controls

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 (British Pharmacopoeia [Veterinary], 1985). 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.

2.2.3. Final product batch test

i)   Sterility

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

ii)   Identity

Identity of culture is performed by Ziehl–Neelsen staining and PCR (see Section B.1.4).

iii)   Safety

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 (British Pharmacopoeia [Veterinary], 1985).

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.

iv)   Batch potency

The potency of johnin is currently determined by chemical assay for protein using a Kjeldahl method. A PPD content of 0.5 ± 0.025 mg/ml of final product is recommended (British Pharmacopoeia [Veterinary], 1985).

2.3. Requirements for regulatory approval

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.

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NB: There are WOAH Reference Laboratories for paratuberculosis
(please consult the WOAH Web site at:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for paratuberculosis

NB: FIRST ADOPTED IN 1991. MOST RECENT UPDATES ADOPTED IN 2021.