Current culture methods for *Mycobacterium avium* subspecies *paratuberculosis*

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**INTRODUCTION**

*Mycobacterium avium* subspecies *paratuberculosis* or *Mycobacterium paratuberculosis* (MAP) is an organism first observed by Johne & Frothingham in 1895. It causes paratuberculosis or Johne’s disease, an intestinal granulomatous infection most often seen among domestic and wild ruminants and has a global distribution. In individual animals, especially from a newly infected farm, a tentative clinical diagnosis must be confirmed by laboratory tests. Confirmation of paratuberculosis depends on the finding of a) either gross lesions with the demonstration of typical acid-fast organisms in impression smears or microscopic pathognomonic lesions and b) the isolation in culture of MAP (Harris and Barletta, 2001; OIE, 2004).

**DIAGNOSTIC TECHNIQUES**

To diagnose the presence of paratuberculosis in an individual clinically suspect animal, a number of laboratory tests can be used including: fecal smears, fecal and tissue culture, DNA probes using feces or tissues, serology, necropsy and histology (OIE, 2004).

*Identification of the agent: bacteriology (microscopy)*

Ziehl–Neelsen-stained smears of feces are examined microscopically. A potential 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, although it is not always possible to distinguish between MAP and other environmental or pathogenic mycobacteria in a fecal smear. A smear may be made from the processed fecal sediment and stained by the Ziehl–Neelsen method or with fluorochrome stain (auramine O or auramine-rhodamine). The presence of single acid-fast bacilli does not indicate a definitive diagnosis since only about one-third of cases can be confirmed on microscopic examination of a single fecal sample (OIE, 2004).

*Identification of the agent: bacteriology (solid media culture)*

MAP infection mainly involves the lower small intestine and adjacent cecum. The MAP organisms are vastly outnumbered by other bacteria in fecal and intestinal tissue specimens. Primary colonies of MAP may be expected to appear any time from 4 to 16 weeks after inoculation on solid media. Primary colonies on Herrold’s egg yolk medium (HEY) containing mycobactin J (MJ) are very small (1 mm in diameter), colorless, translucent and hemispherical. Their margins are round and even, and their surfaces are smooth and glistening. The colonies become more opaque and increase in size (4 or 5 mm) as incubation continues. The colonial morphology changes with age from smooth to rough, and from hemispherical to mammilate (OIE, 2004; Thorel, 1984).

The identification of MAP is based on its requirement for mycobactin and its pathogenicity in the host. Most mycobacteria are able to metabolize mycobactin for themselves. *Mycobacterium paratuberculosis*, *M. silvaticum* and some primary isolates of *M. avium* require mycobactin to grow in the laboratory. Thus, the mycobactin requirement characteristic exists to various degrees within the *M. avium* group (Thorel, 1991). For identification of MAP, small inocula of suspect colonies should be subcultured on the same medium with and without mycobactin, to demonstrate mycobactin dependency. The uncommon, bright yellow pigmented sheep strain is difficult to grow on artificial solid media. It has been reported that unpigmented
sheep strains grow less well than cattle strains, and no cultures should be discarded as negative without prolonged incubation (up to 6 months) (OIE, 2004).

**Decontamination methods**

There are two basic methods in use for the culture of MAP from clinical specimens: (1) oxalic acid and NaOH for decontamination and Löwenstein–Jensen medium for growth, and (2) hexadecylpyridinium chloride (HPC) for decontamination in combination with Herrold’s egg yolk medium (HEY) for growth. Both media contain mycobactin (OIE, 2004).

**Media**

Examples of suitable media are: Lowenstein Jensen medium with MJ, Modified Middlebrook 7H10 with MJ (Australian formula for sheep strain), Middlebrook 7H9 with M J, Becton Dickinson (BD) BACTEC™ 12B culture medium with PANTA antibiotic supplement, MJ & 50% egg yolk supplement, TREK ESP® para-JEM broth with para-JEM GS, AS, EYS and AS w/BLUE, BD BACTEC™ MGIT™ 960 medium with OACC, amphotericin B, naladixic acid, vancomycin (ANV) antibiotic supplement, MJ & 50% egg yolk enrichment, and HEY with MJ and ANV. The advantage of Middlebrook 7H9 and Middlebrook 7H10 media enhanced with MJ is that it is transparent which facilitates the early detection of colonies (Jorgensen, 1982; Merkal, 1970; Payeur et al., 2005; Wiszniewska et al., 2005).

Herrold’s egg yolk medium with MJ and ANV has several ingredients which enhance the growth of MAP and decrease the contamination of other organisms. The egg in Herrold’s medium contributes sufficient phospholipids to neutralize the bactericidal activity of residual HPC in the inoculum. The other media (Middlebrook 7H9 & Middlebrook 7H10) do not have this property. Other treatments can be used for sample decontamination, i.e., oxalic acid at 5%. HPC is relatively ineffective in controlling the growth of contaminating fungi. Amphotericin B enhances the selectivity of the medium by inhibiting contaminating fungi. Nalidixic acid inhibits contaminating gram-negative organisms and vancomycin inhibits contaminating gram-positive organisms. Malachite green is included to help control contaminants and enhance the visibility of colonies. Egg yolk and glycerol provide fatty acids and other nutrients required for the metabolism of mycobacteria (Johansen et al., 2004; Merkal, 1970; OIE, 2004).

**Sample preparation**

Although fecal culture is technically difficult and time-consuming, it is the only test that does not produce false-positive results (100% specificity) for the live animal. It will detect infected animals 6 months or more before they develop clinical signs, and during the clinical stage its sensitivity approaches 100% if shedding (OIE, 2004).

**Processing fecal specimens - Pooled fecal culture using 5 samples per pool**

Because of the cost of individual fecal cultures, pooling methods are being evaluated for the detection of MAP in bovine and ovine feces. Several studies have indicated that pooling feces from herds that contain heavy shedders or have a high prevalence herds is equivalent in sensitivity to culturing individual animals. Pooling feces from herds with low or moderate shedders or in low prevalence herds may fail to detect the organism (Jensen et al., 2005; Pradenas et al., 2005; Ruzante et al., 2005; Wells et al., 2002; 2003; Whittington et al., 2000).

**Homogenization by stirring**

Weigh 2 grams of each sample to be pooled and place into a sterile 50 ml conical centrifuge tube or other suitable vessel. Mix samples by stirring with a sterile wooden stick. If using a 50 ml conical tube, vortex samples vigorously for 10 to 15 seconds until the mixture appears homogeneous. Remove 2 g of the resulting mixture for processing and culture using any of the recommended methods (Payeur et al., 2005).

**Homogenization by stomaching**

Weigh out 2 grams of each sample to be pooled and place into a stomacher bag. Be sure to add the samples to the same corner of the bag to ensure even mixing. Stomach the samples on the highest setting for 2 min. Inspect the mixture to determine if it appears homogeneous. Additional stomaching may be required to homogenize the mixture. Remove 2 g of the resulting mixture for processing and culture using any of the recommended method (Payeur et al., 2005).
Processing fecal specimens – Individual fecal culture
Suspension and decontamination of feces

No refrigerant or chemical preservative is used. If fecal specimens cannot be processed within a week upon receipt at the laboratory, they can be frozen at -70°C to -80°C. Place one centrifuge tube containing 35 ml of sterile distilled water onto a weigh scale located in the Class II Biological Safety Cabinet. Using a sterile wooden tongue depressor, transfer 2 ± 0.1 gm of the fecal specimen into the tube. Mix tube vigorously by shaking for 15 seconds to break up large clumps of fecal matter. Place on commercial shaker for a minimum of 30 minutes, then allow material to sit upright undisturbed at room temperature for a minimum of 30 minutes. With a sterile disposable pipette, transfer 5 ml of supernatant (minimizing fiber transfer) into 25 ml of 0.9% HPC in ½ X BHI broth solution contained in a separate disposable 50 ml conical centrifuge tube. If a refrigerated centrifuge is used, the temperature must be kept above 10°C, as the HPC will precipitate at 4°C. Incubate for 18 to 24 hrs at 37° ± 2°C. After overnight incubation, centrifuge the samples at 900 x g for 30 ± 2 minutes. Discard the supernatant, and resuspend the remaining pellet in 1 ml of antibiotic brew (BHI broth containing 100 µg/ml nalidixic acid, 100 µg/ml vancomycin and 50 µg/ml amphotericin B). Mix the sample by shaking or vortexing vigorously for a minimum of 15 seconds. Incubate the fecal inoculum in the antibiotic brew at 37° ± 2°C for 24 to 72 hours. After the overnight incubation of the fecal inoculum in antibiotic brew, vortex it vigorously for a minimum of 15 seconds (Payuer and Capsel, 2005; Payeur et al., 2005).

Inoculation of culture - solid media

Use Herrold's egg yolk medium (HEY) with mycobactin J (MJ) for the culture of MAP from bovine feces and use Modified Middlebrook 7H10 (MM7H10) with (MJ) for the isolation of MAP from ovine feces. After vortexing the specimens, inoculate four tubes of HEY with MJ and one tube of HEY w/o MJ for bovine specimens, or four tubes of MM7H10 with MJ and one tube of MM7H10 w/o MJ for ovine specimens by pipetting 200-250 µl of the antibiotic brew suspension onto the surface of the media. Rock the tubes to evenly distribute the inoculum. If a liquid culture media (Bactec 12B, MGIT, ESP) is also being used, eliminate one HEY with MJ slant in the setup for both bovine or sheep feces. Slant the inoculated tubes at a 30° angle, ensuring that the caps are loose, and incubate at 37°± 2°C for 5 to 7 days to allow absorption/evaporation of any remaining liquid present on the slant. After all remaining visible liquid has been absorbed, tighten down the caps on the media and continue to incubate upright at 37°±2°C. Examine tubes for growth and contamination at 4, 8, 12 and 16 weeks post-inoculation for bovine samples and at 4, 8, 12, 16, 20 and 24 weeks for ovine samples. Use of a stereomicroscope will facilitate examination of the tubes at 4 weeks. Document all observations (Gwozdz, 2003; Payeur et al., 2005).

Interpretation of results (identification of solid media cultures positive for MAP)

Samples that exhibit bacterial growth typical of MAP on one or more tubes HEY or MM7H10 with MJ, but do not exhibit growth on HEY or MM7H10 w/o MJ at 4, 8, 12 or 16 weeks of incubation (ovine samples can require up to 24 weeks) are considered suspect positive. Typical colonial morphology for MAP is small (1 mm diameter), colorless, translucent and hemispherical, smooth and glistening. The colonies become more opaque and increase in size (ca. 4 mm) and roughness as incubation is continued. The use of a stereomicroscope is highly recommended for earlier detection. Most colonies are visible by 8 weeks after inoculation. Contamination usually increases between 8 – 16 weeks which impedes detection of MAP colonies (Gwozdz, 2003; Payeur et al., 2005).

A single bacterial colony from a HEY or an MM7H10 with MJ should be acid-fast stain tested. Typical cellular morphology of MAP is acid-fast, small (0.5 µm x 1 µm) and generally observed in clusters or clumps. If acid-fast positive, a PCR test should be performed for the presence of the IS900 insertion element. The specimen is considered positive for MAP if growth is observed on one or more tubes of HEY or MM7H10 with MJ, no growth is observed on HEY or MM7H10 w/o MJ, the acid-fast smear is positive and the PCR result is positive.

Variations in the above methods have been described (Collins et al., 1990; Merkal et al., 1968; Shin, 1989; Shin et al., 1990; Singh et al., 1991, Whitlock and Rosenberger, 1990; Whitlock et al., 1991; 2000). The sensitivity of culture may be enhanced using liquid media with centrifugation rather than sedimentation techniques. The double incubation method assists with decontamination of the inoculum (Stabel, 1997). Evaluations have been done using CB18, a zwitertionic detergent which has been shown to increase the
recovery of MAP, but it also has a high level of contamination associated with it (Johansen and Payeur, 2004; Ruzante et al., 2005).

**Liquid media culture**

A more rapid technique for the isolation of MAP from bovine or ovine feces employs the use of a radiometric-based detection system, the BACTEC™ 460. The BACTEC™ 12B medium contains 14C-labeled palmitic acid which is metabolized by *Mycobacterium spp*. The resulting release of radiolabeled CO₂ is measured by the BACTEC™ 460 reader, giving an indication of bacterial growth that is termed the growth index (GI). Samples are decontaminated by a recommended method and inoculated into BACTEC™ 12B medium, incubated at 37°C and read weekly on a BACTEC™ 460 reader for 6 weeks. Any sample with a GI>10 is subcultured or confirmed directly by acid fast staining and PCR. However, as this system is radiometrically based, it is not feasible for use in some laboratories and has been phased out in others (Gwozdz, 2003; Payuer et al., 2005; Whittington et al., 1999).

There are several non-radiometric methods available for the culture of MAP with liquid media. The BD MGIT™ Para TB liquid culture media contains a fluorescent compound (Tris 4, 7-diphenyl-1,10-phenanthroline ruthenium chloride pentahydrate) embedded in silicone in the bottom of the culture tube. This compound is sensitive to the presence of oxygen dissolved in the broth. Initially, the amount of dissolved oxygen present in the broth quenches emissions from the compound and little fluorescence is detected. Later, actively respiring micro-organisms consume the oxygen and diminish the quenching of the fluorescence, allowing it to be detected by the BACTEC™ MGIT™ 960 instrument (Fyock et al., 2005; Payuer et al., 2005; Thomas et al., 2005).

Culture tubes entered into the BACTEC™ MGIT™ 960 instrument are incubated at 37°C for 6 weeks and monitored hourly for increasing fluorescence. Analysis of the fluorescence emitted from each tube is used to determine whether it contains viable organisms. A specific algorithm is used to determine whether the instrument signals positive for a sample for the growth of MAP. A sample is reported as positive if it contains acid-fast bacteria and is determined to be positive for the presence of the IS900 element using PCR (Fyock et al., 2005; Payuer et al., 2005).

Another non-radiometric system is the ESP® Culture System II (TREK Diagnostics) which is based on consumption of gases by viable mycobacteria as part of their metabolic growth cycle. This creates a negative pressure change within the headspace above the broth culture medium within the sealed ESP® bottle. The change in pressure is monitored by a computerized system that signals the user when growth is detected. When the ESP® Culture System II identifies a positive specimen, a red indicator light will be lit on both the outside of the machine and the specific location within a drawer. If the corresponding graph for the specimen indicates a typical Mycobacterium pattern, consisting of a noticeable sharply defined downward curve to the baseline (e.g. “elbow” or “knee” shaped) after approximately 4 or greater days of incubation, the specimen is considered a suspect positive. The specimen is considered positive for MAP if the acid-fast smear is positive and the PCR result is positive for the presence of the IS900 insertion element (Kim et al., 2002; Payuer et al., 2005; Shin, 1989; Shin et al., 1990; van Maanen et al., 2005).

Severe problems were encountered during initial experiments in which these liquid culture methods were tested on fecal samples due to overgrowth by other bacteria (spore forms and fungi); however, these methods have been further developed and are now used with some success in many laboratories (Eamens et al., 2000; Thomas et al., 2005).

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Theme 4: Molecular Biology, Microbiology and Culture

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