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# Decontamination of Bovine Fecal and Environmental Samples Aimed to Culture *Mycobacterium Paratuberculosis* in Liquid Media: A Narrative Review

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**Abstract**

Culture of *Mycobacterium paratuberculosis* (MAP) is the definitive *antemortem* test for the confirmation of paratuberculosis. The culture process involves three steps: decontamination, incubation in a culture medium promoting MAP growth, and identification of MAP, either by phenotypic or genotypic methods. MAP culture can be done either in solid or liquid media, but MAP growth is faster in liquid. However, non-interpretable results due to the overgrowth of other microorganisms are more commonly observed in liquid media. MAP growth and identification are more complicated if other microorganisms overgrow the media. The aim of this review was to assess the methodologic evidence of the effect of decontamination protocols on the growth of irrelevant microorganisms (contamination) in liquid media used in the culture of MAP from bovine fecal and environmental samples.

**Keywords:** Cattle; Contamination; Culture; Johne´s disease

**Abbreviations:** HEYM: Herrold's egg yolk medium; HPC: Hexadecyl pyridinium chloride; MAP: *Mycobacterium paratuberculosis*; NI: Non-interpretable; PCR: polymerase chain reaction; PTB: Paratuberculosis; RMC: radiometric culture VAN: Vancomycin

## First Things First

*Mycobacterium avium* subsp. *paratuberculosis* (MAP) is a slow-growing, mycobactin-dependent, acid-fast bacterium that causes paratuberculosis (PTB), a slow-developing and incurable cattle disease, characterized by chronic granulomatous enterocolitis [1]. The disease causes important economic losses in infected herds [2], varying from 6 to 19% in the production of meat, milk, or both [3]. One difficulty in the control of PTB is the fact that animals are contagious before being clinical (unapparent adult carriers) [4]. Moreover, they may not be detected using the available diagnostic tests [5]. Several tests are available for the *antemortem* detection of MAP-infected animals. These include the detection of MAP antibodies, MAP DNA, or live organisms by bacterial culture [6,7]. Sensitivity and specificity for the antemortem diagnosis of PTB vary significantly depending on MAP infection stage and intrinsic characteristics of each test [4]. The bacterial culture of MAP has a reported sensitivity between 30 and 70%. The specificity is considered almost 100% if a molecular method such as polymerase chain reaction (PCR) is used to confirm MAP identification [8].

The culture process is based on the general principles of mycobacterial culture: Decontamination (to reduce the number of other microorganisms), incubation in a culture medium promoting MAP growth, recognition of MAP colonies or a particular sign of growth, and phenotypic or genotypic methods [9]. In addition, it can be done either on solid or liquid media. Solid media are less expensive and require less instrumentation. However, MAP growth is slower (10 to 20 weeks) compared to liquid media (8 to 12 weeks). Liquid media is more sensitive but phenotypic and/or genotypic methods are required (i.e., differential staining or PCR) [10]. On the other hand, it has had suggested that MAP culture in liquid media has superior analytical and diagnostic sensitivity than solid media-based protocols [5,9]. However, up to 60% of MAP liquid cultures have been reported to be mixed cultures or contain

contaminants, leading to non-interpretable (NI) results [9]. On this basis, the sensitivity of the culture (both liquid and solid) is imperfect because of the stage of disease, sample selection, and sample decontamination [10].

The contamination rates in MAP-culture reports are often defined as the proportion of culture samples that contained organisms other-than MAP [11], whether they affect the identification of MAP by subsequent techniques. A key component of the MAP-culture process is an optimal method of decontamination. This means that the decontamination process has the least inhibitory effect on MAP growth but effectively eliminates most if not all undesirable organisms. As an introductory final point to state, fecal and environmental samples present a particular challenge due to their high load of enteric bacteria and biological components [9]. Moreover, there is sample variation according to the diet and the geographical location of the livestock, further complicating the scenario [9,11].

## MAP Isolation Protocols

MAP was first isolated in 1910. Since then, differences to the original culture method have been implemented. Among other difficulties, the need for a prolonged incubation period to culture it has led to several studies searching for the most suitable combination of decontaminants and antimicrobials that will efficiently inhibit bacterial and fungal contaminants, without affecting the growth of the primary MAP isolate. Several decontamination protocols exist and are described in the literature. All of them have in common sedimentation, incubation, and/or centrifugation. Comparative advantages and disadvantages are described below. With the intention of keeping the line of our finding procedure based-decontamination protocols are going to be explored in the following order: sedimentation, double incubation, and double centrifugation-based protocols. When comparing fecal culture techniques on solid media, conventional sedimentation procedures gave similar results to those using centrifugation with double incubation and antibiotics (Cornell method) or centrifugation alone [12]. On the contrary, increased sensitivity of detection by 3-fold when compared centrifugation-based to the sedimentation method has been also reported [13]. Double incubation-based protocols using RMC seem to be more sensitive than sedimentation and Herrold´s egg yolk medium (HEYM) culture when used in young animals, is expected to shed small amounts of MAP [14]. In addition, incubation temperature has an impact on decontamination results, concluding that double incubation at 42˚C was more selective and sensitive than the standard procedure at 37˚C on this matrix (fecal slurry) [15].

Double centrifugation-based protocols increase the chances of detecting animals shedding small numbers of organisms [16]. Recent approaches concluded that the most successful method for bovine fecal sample processing involves double centrifugation, and then the culture in two stages-culture systems (liquid, then solid). The contamination rate was slightly higher for the two-stage method than for HEYM. According to the author, this can be due to the centrifugation process and to the longer storage of the samples [17]. Nevertheless, other authors have reported a significant increase in contamination (60 vs. 26%) in fecal samples prepared for culture by the centrifugation method compared to those processed by the sedimentation method in HEYM cultures, with overall detection rates being similar between methods [16]. Other authors have described an alternate centrifugation/double incubation method, with lower centrifugation speeds than the Cornell method, to reduce contamination problems. In their experience, centrifugation increased culture sensitivity up to threefold in cattle shedding low numbers of MAP compared with sedimentation [18]. Evidence in this regard reinforces and extend several reports on concentration methods using centrifugation with or without double incubation are more sensitive compared to those based on sedimentation (from 39 to 68%) [5] since laboratories employing centrifugation methods had increased detection of positive samples (19%) compared to those using sedimentation as decontamination protocol (15%). Double incubation-centrifugation has been found to be the most sensitive for RMC of MAP (89%), even more than procedures involving sedimentation to solid media, filter concentration to RMC, or a modified version of the method used in their study [18]. In a small report, the centrifugation/double incubation method [19] combined with RMC showed promising results that appeared superior to sedimentation. In the same report, authors mentioned that this method was possibly superior to filter concentration with RMC, independent of the selection of antibiotics or the culture step, without further details. Accordingly, it has been reported that the filter concentration of MAP, although relatively successful, did not result in as high a recovery rate as the centrifugation method and was deemed technically unsuitable for application to large numbers of samples [17]. On the other hand, marked or minor advantages of centrifugation/double incubation procedures over filter concentration, depending on the method used have been reported [5]. The centrifugal concentration of bovine fecal specimens has been shown to shorten the incubation time required. However, centrifugation did not increase the isolation rate of MAP from fecal specimens when compared to the standard sedimentation method [16].

## Other Factors that could Affect MAP Recovery Rates

Considering the relationship between the effectiveness of decontamination and the success of isolation of MAP, we reported the contamination rate as well as MAP isolation rate. Both should be included as part of the methodological assessment in MAP-related studies. Several reasons within decontamination steps may explain the variability in reported estimates (both contamination and MAP recovery rate) from liquid cultures aiming to detect MAP, as follows.

### Sample quantity

No differences have been reported or mentioned in the


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literature, related to sample quantity. Nevertheless, the amount of matrix cultured (feces, environmental sources) should be standardized, as other researchers have shown previously that it can influence the contamination rate. The use of 2 g of fecal samples rather than 1 g along with the Cornell decontamination method (double incubation-centrifugation) on HEYM or BACTEC™ 12B cultures has been reported to improve the detection of sub clinically MAP-infected animals [20]. There is no information on the effect of sample quantity in the liquid culture of MAP, as there is some for the solid alternative, as it is mentioned above. It seems to affect but no approach has been made so far.

### Chemical decontamination

Methods for the reduction of bacterial and fungal contamination-including oxalic acid, NaOH, sodium hypochlorite, phenol, benzalkonium chloride, and hexadecyl pyridinium chloride (HPC), have been evaluated, emphasizing greatly the latter two decontaminants [20]. A report concluded that longer double incubation times (24 and 48 h) in HPC and *vancomycin* (VAN) were positively related to lower contamination in radiometric culture (RMC) systems ([5]. However, other authors have reported no effect of HPC for up to 5 days [21].

### Pellet handling

Different antibiotics have been studied a single or combined (e.g., PANTA™ Plus, VAN, combination of penicillin, chloramphenicol, and amphotericin B) addition to determining the optimal concentration (maximal effect on contaminating microorganisms but a minimal influence on the mycobacteria. Separately from their inhibitory effect on contaminating bacteria and fungus, these components can also bring a negative influence on the growth of the mycobacteria, which is dependent on the strain (both C and S) and antibiotic concentration [22].

### Inoculation of media and the media components

The commercially available liquid media, BACTEC™ 12B (Becton, Dickinson), based on Middlebrook 7H9 has been the one most widely applied in veterinary contexts. The system supports the growth of the two major strain types of MAP [23]. Losses of $1_{log}10$ from a 1 mL volume due to the final aliquots of 100 µL for inoculation of BACTEC™ 12B cultures, when sheep feces were analyzed have been reported [24]. The authors established that 1 mL is the minimal practical volume for resuspension of the pellet resulting from about 2 g of sheep feces. Using more than 100 µL for inoculation of BACTEC™ 12B vials may dilute the concentration of substrates necessary for the growth of MAP S strain. Studies on this respect for MAP C strain are needed.

## Aspects to Consider and Research Approaches Needed

A few reasons were identified to explain the variability observed in the reported estimates of contamination rate and MAP recovery rate performed in liquid media using fecal or environmental samples. A large proportion of the overall variance (heterogeneity) is most likely associated with individual culture protocol differences. The available information about the optimal decontamination protocol is insufficient according to our results. The culture media contamination phenomena are clustered by the origin of samples, season, and environmental factors [9]. The author of this review suggests separating the research approaches to adapt an optimal decontamination protocol according to each matrix. A research approach looking for the proper combination of antimicrobials that will effectively inhibit bacterial and fungal contaminants with the least effect on the growth of the primary isolate is justified. Changes in VAN (being the one most used) concentration or duration of exposure must then, be considered. The double incubation, centrifugation, one-step HPC-water methodology must be considered for further analysis since non-contamination was reported (defined as “negligible” by the authors) [25].

The use of a wide array of decontamination protocols, which are compared against an equally wide array of reference protocols, makes a comparison of results from different studies difficult. Therefore, there is still a need on further research on the standardization of the protocols since there seems to be a lack of continuity in the method of isolation of MAP from submitted samples at the laboratory level. This void directly affects reports all over the world, leading to incomparable, unrepeatable, and undiscussable results, since culture is still considered the gold-standard test for the diagnosis of PTB [26]. Contamination by irrelevant microorganisms can reduce the diagnostic sensitivity of the culture and increase the complexity and cost of confirming the presence of MAP [11]. The detection of other-than MAP mycobacteria by the RMC presents a disadvantage to the routine processing of samples, because of the added cost of subculture into an additional RMC vial and PCR confirmation and a failure to obtain a result for MAP due to the contamination [23].

There is a lack of consistency in the MAP culture-related literature about the meaning of “contamination”, which makes difficult the comparison between different studies. Contamination can refer to a mixed culture of MAP and other-than MAP microorganisms, a light growth of irrelevant microorganisms, or to a complete overgrowth of the medium [9]. Moreover, many variations are reported in decontamination protocols and culture media [11]. The reported contamination rates for MAP in RMC are extremely variable. The lack of case definition when culture contamination is reported, prevents comparisons between studies [10]. When a growth signal is detected in the BACTEC™ 12B or any other RMC system, additional tests must be performed to confirm the presence of MAP. Thus, the cost of culture is significantly increased by the presence of irrelevant microorganisms that generate a positive signal. Furthermore, the chance of detecting MAP in the presence of other organisms may be reduced, as has been reported with PCR [11]. Therefore, NI results should always


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be reported with MAP culture results to allow the reader to consider this information while interpreting the results.

## A Concluding and Daring Thought

According to this review results, it seems to be recommendable to use 2 g of the matrix (bovine fecal and environmental samples), then mix with saline/water solution, and later use 0.75% HPC + VAN as the decontamination solution for no less than 24 hours, and finally filter the decontaminated solution using a 13-mm-diameter, 3 µm-pore-size filters prior to incubation. In addition, seems like the double incubation, centrifugation (one-step HPC-water) protocol has shown the best results on MAP recovery and contamination rate. Nevertheless, it is important to consider the information with the prudence that it deserves and to consider that the outcomes of interest (MAP recovery and contamination rate) may vary from case to case. Information from the scientific literature is limited, because of study's objectives, designs, and reporting. The author considers that the use of, in some cases standardized and, in some others, varied protocols make a direct comparison between studies tedious. Future studies in this area should follow standardized guidelines when designing and implementing studies and reporting their results.

## References


1. Harris NB, Barletta RG (2001) *Mycobacterium avium* subsp. paratuberculosis in Veterinary Medicine. Clin Microbiol Rev 14(3): 489-512.
2. Nielsen SS, Toft N (2008) *Antemortem* diagnosis of paratuberculosis: A review of accuracies of ELISA, interferon-γ assay and faecal culture techniques. Vet Microbiol 129(3-4): 217-235.
3. McKenna SLB, Keefe GP, Tiwari A, VanLeeuwen J, Barkema HW (2006) Johne's disease in Canada part II: Disease impacts, risk factors, and control programs for dairy producers. Can Vet J 47(11): 1089-1099.
4. Fecteau M, Whitlock R (2010) Paratuberculosis in Cattle. In: Paratuberculosis: Organism, Disease, Control. CAB International Oxfordshire England pp: 144-156.
5. Eamens GJ, Whittington RJ, Marsh IB, Turner MJ, Saunders V, et al (2000) Comparative sensitivity of various faecal culture methods and ELISA in dairy cattle herds with endemic Johne's disease. Vet Microbiol 77(3-4): 357-367.
6. Nielsen SS (2010) Immune-based diagnosis of paratuberculosis. In: Paratuberculosis: Organism, Disease, Control. CAB International Oxfordshire England pp: 284-293
7. Stevenson K (2010) Diagnosis of Johne's Disease: Current limitations and prospects. Cattle Pract 18: 104-109.
8. Van Schaik G, Pradenas FM, Mella NA, Kruze VJ (2007) Diagnostic validity and costs of pooled fecal samples and individual blood or fecal samples to determine the cow- and herd-status for *Mycobacterium avium* subsp. paratuberculosis. Prev Vet Med 82(1-2): 159-165.
9. Whittington RJ, Marsh IB, Saunders V, Grant IR, Juste R, et al. (2011) Culture phenotypes of genomically and geographically diverse *Mycobacterium avium* subsp. paratuberculosis isolates from different hosts. J Clin Microbiol 49(5): 1822-1830.
10. Whittington R (2010) Cultivation of *Mycobacterium avium* subsp. paratuberculosis. In: Paratuberculosis: Organism, Disease, Control. CAB International Oxfordshire England pp: 244-266.
11. Whittington RJ, Begg DJ, de Silva K, Purdie AC, Dhand NK, et al. (2017) Case definition terminology for paratuberculosis (Johne's disease). Vet Res 13: 328.
12. Whittington RJ (2009) Factors affecting isolation and identification of *Mycobacterium avium* subsp. paratuberculosis from fecal and tissue samples in a liquid culture system. J Clin Microbiol 47(3): 614-622.
13. Whipple DL, Kapke PA, Andersen PR (1992) Comparison of a commercial DNA probe test and three cultivation procedures for detection of *Mycobacterium* paratuberculosis in bovine feces. J Vet Diagn Invest 4: 23-27.
14. Whitlock RH, Bruce JB, Spencer PA, Hutchinson LJ (1988) Proceedings of the 69th Conference of Research Workers on Animal Disease p: 24.
15. McDonald WL, Ridge SE, Hope AF, Condron RJ (1999) Evaluation of diagnostic tests for Johne's disease in young cattle. Aust Vet J 77(2): 113-119.
16. Perry GH, Vivanco H, Holmes I, Gwozdz JM, Bourne J (2006) No evidence of *Mycobacterium avium* subsp. paratuberculosis in vitro produced cryopreserved embryos derived from subclinically infected cows. Theriogenology 66(5): 1267-1273.
17. Kim YG, Bech-Nielsen S, Gordon JC, Slemons RD, Spangler E (1989) Comparison of two methods for isolation of *Mycobacterium* paratuberculosis from bovine fecal samples. Am J Vet Res 50(7): 1110-1113.
18. Ridge SE (1993) Cultivation of *Mycobacterium* paratuberculosis from bovine fecal samples by using elements of the Roche MB Check system. J Clin Microbiol 31(2): 400-405.
19. Whitlock RH, Rosenberger AE (1990) Fecal culture protocol for *Mycobacterium* paratuberculosis: A recommended procedure. In: Proceedings of the 94th US Animal Health Association, Denver, CO pp: 280-285.
20. Shin SJ (1989) New methods for reduction in bacterial and fungal contamination from fecal culture for *Mycobacterium* paratuberculosis. In: Report of the committee on Johne's disease. Proceedings of the 93rd annual meeting of the US Animal Health Association, Las Vegas pp: 381.
21. Stabel JR (1997) An improved method for cultivation of *Mycobacterium* paratuberculosis from bovine fecal samples and comparison to three other methods. J Vet Diagn Invest 9(4): 375-380.
22. Whipple DL, Merkal RS (1983) Modifications in the techniques for cultivation of *Mycobacterium* paratuberculosis. Proc Int Colloq Res Paratuberculosis Ames IA p: 82-92.
23. Gumber S, Taylor DL, Whittington RJ (2007) Protein extraction from *Mycobacterium avium* subsp. paratuberculosis: Comparison of methods for analysis by sodium dodecyl sulphate polyacrylamide gel electrophoresis, native PAGE and surface enhanced laser desorption/ionization time of flight mass spectrometry. J Microbiol Methods 68(1): 115-127.
24. Collins MT (1996) Diagnosis of paratuberculosis. Vet Clin North Am Food Anim Pract 12(2): 357-371.
25. Reddacliff LA, Whittington RJ (2003) Experimental infection of weaner sheep with S strain *Mycobacterium avium* subsp. paratuberculosis. Vet Microbiol 96(3): 247-258.
26. Whittington RJ, Whittington AM, Waldron A, Begg DJ, de Silva K, et al. (2013) Development and validation of a liquid medium (M7H9C) for routine culture of *Mycobacterium avium* subsp. paratuberculosis to replace modified Bactec 12B medium. J Clin Microbiol 51(12): 3993-4000.
27. Gilardoni LR, Paolicchi FA, Mundo SL (2012) Bovine paratuberculosis: a review of the advantages and disadvantages of different diagnostic tests. Rev Arg Microbiol 44: 201-215.




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