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ORIGINAL ARTICLE

Decontamination protocols for bovine fecal and environmental samples for culture of *Mycobacterium avium* subsp. *paratuberculosis* growth on solid media

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ABSTRACT. Bacteriological culture of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is considered the gold standard to confirm its presence in several matrices for Johne's disease diagnosis. Whether it is a liquid or solid culture, a problem with MAP culture is that non-interpretable results arise because of overgrowth by other microorganisms, making MAP growth and identification more difficult or impossible. We systematically reviewed published decontamination protocols and their effects on MAP culture from bovine fecal and environmental samples on solid media. Based on our findings, we suggest a step-by-step decontamination protocol. The OVID®/MEDLINE, PubMed®, SciELO Citation Index®, and Redalyc® platforms as well as the International Colloquium on Paratuberculosis (ICP) proceedings and the reference lists were reviewed to identify relevant studies. The inclusion criteria considered articles published in English, Portuguese, French, German, Spanish, and peer-reviewed journals. The exclusion criteria included unrelated topics, species other than bovines, other than environmental/faecal samples, other than diagnostic techniques of interest, and non-original articles. Definitive studies were obtained through the authors' consensus regarding their eligibility and quality. In total, 1,004 publications matched the search terms, and 27 articles met the inclusion criteria, of which 45 derived and reported 15 different decontamination protocols. The centrifugation-one-step hexadecylpyridinium chloride protocol, which used over 22,154 fecal samples in three studies, was found to be the most suitable, reporting an average MAP isolation rate of 3.99% (886/22,154) and an average contamination rate of 0.17% (38/22,154). This systematic review highlights the need for further refinement of decontamination protocols to minimize the loss of viable MAP during processing of bovine fecal and environmental samples.

Keywords: Cattle, culture, HEYM, Johne's disease, overgrowth, paratuberculosis.

INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP) is a slow-growing, mycobactin-dependent, acid-fast bacterium that causes paratuberculosis (PTB), a slow-developing, incurable cattle disease (Sweeney, 1996). MAP infection is characterized by chronic granulomatous enterocolitis, which occurs after a long and variable incubation period (Clarke, 1997; Harris & Barletta, 2001). This disease causes significant economic losses in infected herds (Nielsen & Toft, 2011), such as decreased milk production, decreased slaughter value, and premature culling. The estimated loss varies from 6 to 19% in the production of meat, milk, or both (McAloon *et al*., 2016; Shephard *et al*., 2016), and the average annual losses in major dairy-producing regions worldwide have been estimated at US$33 per cow, or ~1% of gross milk revenue (Rasmussen *et al*., 2021). In addition, zoonotic potential has been proposed since MAP has been frequently found in humans with Crohn's disease (Eltholth *et al*., 2009; Waddell *et al*., 2015).

Worldwide disease control is based on herd testing and strategic changes in herd management practices (Field *et al*., 2022). One difficulty in PTB control is that animals are infectious before being clinically infected or diseased (subclinically infected animals) (Fecteau & Whitlock, 2010). Moreover, subclinically infected animals may not be 100% detected using the available diagnostic tests (Sweeney *et al*., 2012).

Several tests are available for MAP diagnosis. These include tests for the pathogen's detection (culture or direct PCR of feces, tissues, or milk), tests for the host's immune response (antibody detection ELISA on serum or milk, various assays for cell-mediated immunity such as delayed-type hypersensitivity tests), or tissue inflammatory response (gross pathology and histopathology) (Nielsen & Toft, 2008; Stevenson, 2010). The sensitivity and specificity of tests for the diagnosis of PTB vary significantly depending on the MAP infection stage and intrinsic characteristics of each test. Sensitivity estimates for the bacterial culture of MAP from feces range from 16 to 74% across species and stages of disease (Nielsen & Toft, 2008; Whittington *et al*., 2017), and its specificity is considered to be almost

100% if a confirmation test, such as polymerase chain reaction (PCR), is used to confirm MAP isolation (Tavornpanich *et al*., 2008; Whittington *et al*., 2011).

MAP can be cultured on either liquid or solid media. Liquid culture methods have a higher analytical and diagnostic sensitivity than solid medium, and growth can be detected sooner, but a formal identification of MAP by a molecular method is required, making the identification of MAP more difficult and expensive (Eamens *et al*., 2000; Whittington, 2009; Whittington *et al*., 2017). However, identification of the organism is more difficult in liquid culture because the appearance of colonies and mycobactin dependence are not observable, and the growth of other organisms needs to be ruled out. In addition, such organisms are more sensitive to recovery than MAP C strains because the ability of solid media to support their growth is well established (Whittington, 2009).

The MAP microbiological culture process is based on the general principles of mycobacterial culture procedures: 1) decontamination to reduce the number of other microorganisms; 2) incubation in a culture medium that promotes MAP growth; 3) recognition of MAP colonies on solid media or a particular sign of growth in liquid media; and 4) MAP identification either by phenotypic or genotypic methods (Merkal *et al*., 1964; Dane *et al*., 2022).

Culture contamination can be due to the light growth of irrelevant microorganisms (IMs), a MAP-mixed culture, or to a complete overgrowth of the medium, hindering the growth and identification of MAP. However, precautions must be taken to prevent contamination by IMs, as this can reduce the diagnostic sensitivity of culture and increase the complexity and cost of confirming the presence of MAP (Whittington, 2009; Dane *et al*., 2022). Such scenarios reveal the importance of defining a decontamination process that is sufficient to prevent IMs and allow MAP detection. Moreover, a significant amount of dispersed and relatively inconsistent information on MAP decontamination protocols and their effect on the excessive growth of IMs is available worldwide. Therefore, this study aimed to systematically review the literature on decontamination protocols and their effect on the excessive growth of IMs or contamination in solid media used in the bacteriological culture of MAP from bovine fecal and environmental samples, and to suggest a decontamination protocol, according to our results.

MATERIAL AND METHODS

This systematic review (SR) was designed, performed, and reported in accordance with the PRISMA guidelines, as suggested by Page *et al.* (2021). An a priori established and pre-tested SR protocol was carried out, including the study question, procedure for literature search, study inclusion/exclusion criteria, checklists for conducting relevance screening, basic characterization, methodological assessment, and data extraction on relevant primary research.

Throughout this SR, we refer to a citation or article (depending on the phase of the systematic information analysis process) as an entire paper, publication, or research report, and to a study as the group of samples collected with a particular purpose within the citation or article.

Search strategy

The primary search was conducted on December 10, 2022. The process of identifying relevant articles considered a specific research question: How do decontamination protocols affect the growth of microorganisms other than MAP-irrelevant microorganisms in solid media cultures in bovine fecal and environmental samples? Four databases were searched (i.e., OVID®/MEDLINE, PubMed®, SciELO Citation Index®, and Redalyc®). The proceedings from the 3rd (1991) to the 12th (2014) International Colloquium on Paratuberculosis (ICP) were available from the platforms explored and therefore reviewed. The 13th and 14th proceedings (2016 and 2018, respectively) were available on the International Association for Paratuberculosis website. This last material was hand-searched for existing published primary studies. In addition, references related to the SR subject were manually searched in Behr & Collins (2010) and Behr *et al.* (2020) books to track primary publications, as well as in two previous reviews on the topic (Dane *et al*., 2022; Field *et al*., 2022).

The topic was divided into components, and the search terms used to find relevant studies on the platforms were (*mycobacterium avium paratuberculosis* OR paratuberculosis OR johne*) AND (cattle OR bovine? OR cow OR dair* OR beef OR heifer? OR bull? OR calf OR calves OR environment* OR livestock) AND (faec* cultur* OR fecal cultur* OR bacteri* cultur* OR bacteri* isolat* OR microb* cultur* OR microb* isolat* OR environment* cultur* OR cultivation) AND (contamina* OR decontamina* OR irrelevant? OR microorganism? OR irrelevant bacteri* OR fung* grow* OR overgrow*).

Eligibility screening

The inclusion criteria considered only original articles published in English, Portuguese, French, German, and Spanish, and in peer-reviewed journals. No publication year or country limitation was considered. In the case of ICP proceedings and other abstracts found, studies were included in the search strategy only to identify further citations already published in peer-reviewed journals. The first selection of publications was performed according to the information contained only in the title. Two of the authors performed the selection, and the Kappa coefficient was estimated. The inclusion of citations was performed considering the possibility of answering our investigation question. The reasons for not inclusion were as follows: i) irrelevant topics (e.g., coronavirus, *Staphylococcus*, *Salmonella*, chemotherapy, Crohn's disease, economic impact, control programs, *Mycobacterium bovis*, vaccine); ii) other-than bovines (e.g., goats, sheep, human, pigeon); iii) other-than environmental/fecal

samples (e.g., milk, tissue, cheese); iv) other-than diagnostic technique of interest (e.g., liquid media, PCR, ELISA, RFLP, radiometric culture, AGID, PMS-phage assay); v) not an original article (e.g., review, book). Duplicated articles were not considered. All the citations selected by at least one of the three authors were considered to continue the process.

Two authors screened eligible articles using the abstract. The Kappa coefficient was then calculated. The inclusion and exclusion criteria were the same as those for title screening. Conflicting was resolved through consensus between reviewers and if necessary, by a third reviewer. The remaining articles were studied by two authors using the entire paper to ensure that they contained relevant information (evidence) to answer the question. The Kappa coefficient was then calculated. Each full text was reviewed with particular attention to the materials and methods, and results sections. Articles were considered eligible if the following criteria (in addition to the conditions mentioned above for title and abstract screening) were assessed: 1) describes or cites a primary source of the decontamination protocol(s), and 2) reports contamination rate. Conflicting was resolved through consensus between reviewers and if necessary, by a third reviewer.

Two authors manually searched the reference lists of relevant articles identified by full-text screening for additional published primary articles (snowballing).

ICP proceedings and other abstracts identified during the primary search were revised to identify further citations in peer-reviewed journals. In this regard, abstracts that were able to answer the research question were identified, and an email was sent to the corresponding author (or other available) to inquire if the study related to such an abstract had been subsequently published in a peer-reviewed journal. The articles obtained from this step, as well as those detected by Behr & Collins (2010) and Behr *et al*. (2020), were screened by two of the authors.

Data extraction

After all available articles were compiled, a descriptive summary was prepared in the form of a large summary table, taking into consideration bibliometric information, decontamination protocol, contamination rate, MAP detection rate, and other relevant findings about the question of interest.

RESULTS

The electronic search, combining results from both search engines and removing duplicate references, yielded 1,004 eligible citations potentially related to the subject of this SR. The 13th and 14th proceedings (2016 and 2018, respectively) provided 17 abstracts found by hand searching, but none progressed to the next phases of the SR. Citations to be screened were published between 1949 and 2022.

After reading the titles, 764 were considered irrelevant (consented by two authors). The final number of citations obtained by title screening was 240 (retained by at least one reviewer). After reading the abstracts of the articles, 78 were excluded (by both authors) and 162 original articles remained for full-text review. Twenty-seven articles were completely reviewed by full-text and kept for data extraction, after dismissing 96 articles because the decontamination protocol and/or contamination and/or MAP-recovery rates were not available in sufficient detail.

The snowballing strategy was then applied to the reference lists of the 27 definitive articles, and no more citations were found. In addition, the same strategy was applied to books and reviews, and no more citations were found. The final number of articles fulfilling the eligibility criteria and hence included in the qualitative synthesis was 27. Figure 1 describes the SR protocol and the selection of relevant articles.

All articles were written in English, except for one each in German and Spanish. The first relevant publication was published in 1972 and the most recent in 2017. Relevant citations were published in 21 journals.

Table 1 presents the general information extracted from the relevant articles concerning the research question, including results in terms of overgrowth of the solid media used and MAP-recovery. A total of 45 studies were derived from the relevant articles, reporting the use of 15 different decontamination protocols applied to 41,847 bovine fecal and environmental samples cultured in solid media, considering each pool as an individual sample, because the estimates of contamination and recovery of MAP are also individualized.

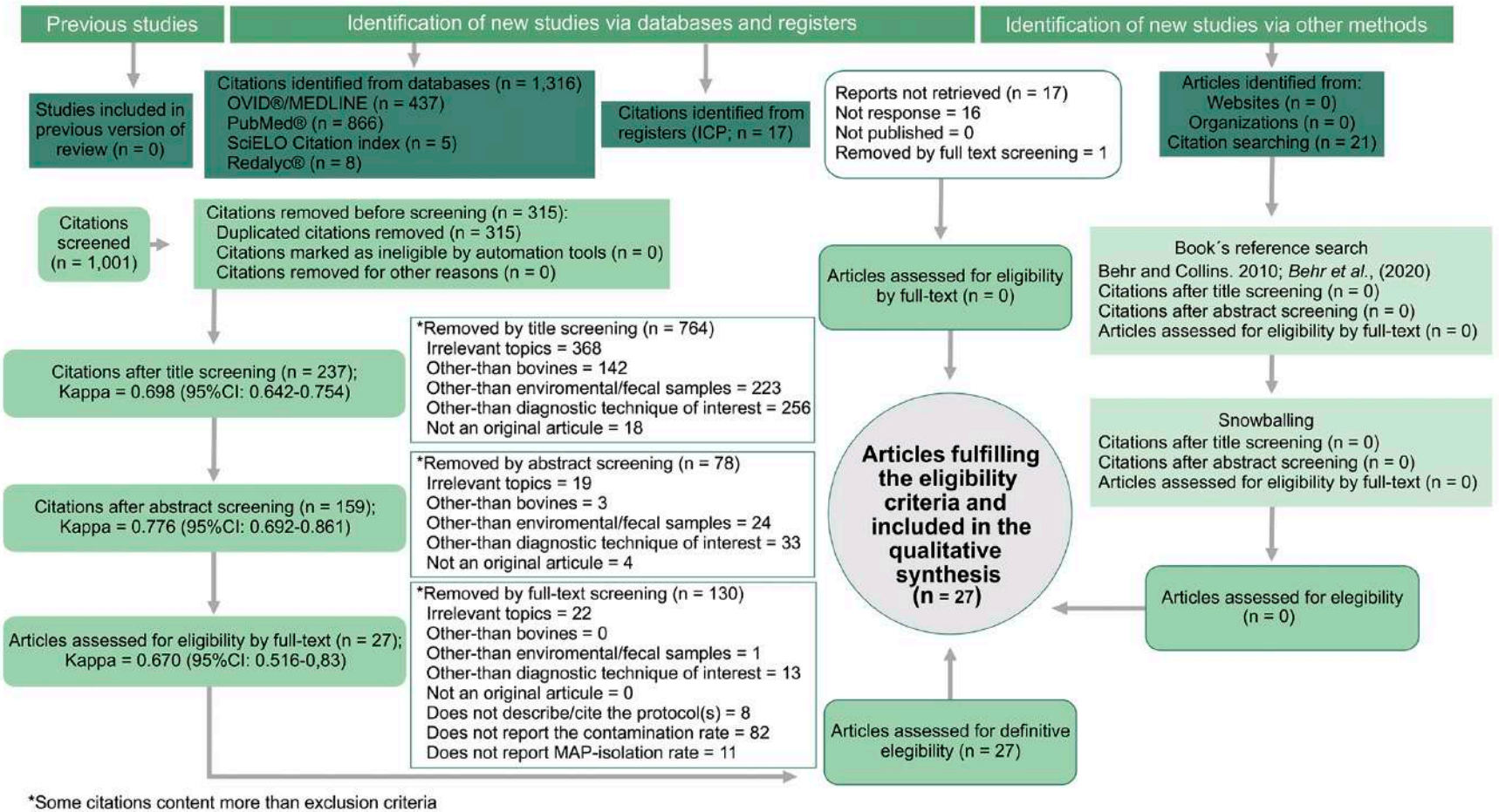

Figure 1.
Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow chart describing the progress of citations in a systematic review.

Table 1.
General information extracted from relevant articles concerning the research question.

Reference	Decontamination protocol (source, if reported)	Study number	Number of samples+	MAP-isolation rate (%)	Final contamination rate (%)
Merkal & Richards (1972)	Sedimentation-one-step BAC	Study 1	788 (I)	5.5	13.7
Jorgensen (1982)	Sedimentation-centrifugation-one-step NaOH/OA (Beerwerth 1967)	Study 1	1,413 (I)	31.9	15.4
		Study 2		41.7	2.6
Kim *et al.* (1989)	Sedimentation (Whipple & Merkal 1983)	Study 1	131 (I)	43.8	25.9
	Centrifugation-one-step HPC-water (Turcotte *et al.* 1986)			49.6	60.3
	Sedimentation (Whipple & Merkal 1983)	Study 2		NR*	25.9
	Centrifugation-one-step HPC-water (Turcotte *et al.* 1986)				60.3
	Sedimentation (Whipple & Merkal 1983)	Study 3		38.8	27.5
	Centrifugation-one-step HPC-water (Turcotte *et al.* 1986)			44.6	37.4
	Sedimentation (Whipple & Merkal 1983)	Study 4		NR**	27.5
	Centrifugation-one-step HPC-water (Turcotte *et al.* 1986)				37.4
McNab *et al.* (1991)	Centrifugation-one-step HPC-water	Study 1	2,943 (I)	0.13	19.2
Whipple *et al.* (1992)	Sedimentation-one-step HPC-water (Cornell method)	Study 1	170 (I)	16.7	5.3
	Centrifugation-one-step HPC	Study 2		18.8	15.9
	Double-incubation-centrifugation-one-step HPC (Cornell method)	Study 3		15.9	0
Stabel (1997)	Sedimentation-one-step HPC-water	Study 1	24 (I)	56.5£	75
				60.9 ££	
	Centrifugation-one-step HPC	Study 2		47.8£	100
				41.7££	
	Double-incubation-centrifugation-one-step HPC (Cornell method)	Study 3		45.8£	0
				51.2 ££	
	Double-incubation-centrifugation-one-step HPC (NADC method)	Study 4		66.7£	20
				83.3££	
Reichel *et al.* (1999)	Sedimentation-centrifugation-one-step HPC-water	Study 1	450 (I)	23	5.8
McDonald *et al.* (1999)	Sedimentation-one-step HPC	Study 1	168 (I)	0	4
	Double-incubation-double centrifugation-one-step HPC-water (modified from Whitlock & Rosenberger 1990)	Study 2	210 (I)	6.2	16
Kalis *et al.* (1999)	Sedimentation-centrifugation-NaOH/OA (Beerwerth 1967)	Study 1	733 (I)	5.9‡	22,6
				63.6‡‡	
		Study 2	151 (P)	18.5‡	19.9
				72.7‡‡	
Kalis *et al.* (2000)	Sedimentation-centrifugation-NaOH/OA (Beerwerth 1967)	Study 1	2,989 (I)	5	7,04
	Sedimentation-centrifugation-NaOH/OA (Beerwerth 1967)	Study 2	59 (I)	72.9†	0
				78††	
Eamens *et al.* (2000)	Sedimentation-one-step HPC-water	Study 1	179 (I)	8.4	0
	Double-incubation-centrifugation-one-step HPC-water (Whitlock & Rosenberger 1990)	Study 2		14.5	0.6
Soto *et al.* (2002)	Double-incubation-centrifugation-one-step HPC-water	Study 1	250 (I)	16	7.6
Nielsen *et al.* (2002)	Sedimentation-centrifugation-NaOH/OA (Beerwerth 1967)	Study 1	3,125 (I)	4.6	14.8

Reference	Decontamination protocol (source, if reported)	Study number	Number of samples+	MAP-isolation rate (%)	Final contamination rate (%)
Huda *et al.* (2003)	Sedimentation-centrifugation-NaOH/OA (Beerwerth 1967)	Study 1	371 (I)	4	23
Muskens *et al.* (2003)	Sedimentation-centrifugation-NaOH/OA (Beerwerth 1967)	Study 1	422 (I)	17.3	16.5
Sorensen *et al.* (2003)	Double-incubation-centrifugation-one-step HPC-water (Stabel 1997)	Study 1	500 (P)	3.4	6
Glanemann *et al.* (2004)	Sedimentation-centrifugation-NaOH/OA (Beerwerth 1967)	Study 1	1,144 (I)	8.1	17.6
	Double-incubation-centrifugation-one-step HPC (Shin 1989; Whitlock & Rosenberger 1990)	Study 2		1.6	21.5
Nielsen *et al.* (2004)	Sedimentation-centrifugation-NaOH/OA (Beerwerth 1967)	Study 1	2,513 (I)	3.3	13.2
		Study 2		6.9	14.6
Soumya *et al.* (2009)	Double-incubation-centrifugation-one-step HPC-water (OIE 2004)	Study 1	40 (I)	52.5	10
Gao *et al.* (2009)	Double-incubation-centrifugation-one-step HPC (Whipple *et al.* 1991; Stabel 1997)	Study 1	110 (I)	41.8	0.9
Fernández-Silva *et al.* (2011)	Centrifugation-one-step HPC (according to FLI 2007)	Study 1	36 (P)	5.7	8.6
		Study 2	1 (P)	100	0
Laurin *et al.* (2015)	Double centrifugation-one-step HPC (Stabel 1997)	Study 1	345 (I)	45.6	9.3
Donat *et al.* (2015)	Centrifugation-one-step HPC (according to FLI 2012)	Study 1	200 (I)	14.5	14
Donat *et al.* (2016)	Centrifugation-one-step HPC (according to FLI 2012)	Study 1	22,057 (I)	4	0.07
Heuvelink *et al.* (2017)	Centrifugation-one-step HPC (according to FLI 2010)	Study 1	61 (I)	1.6	30
	Sedimentation-centrifugation-NaOH/OA (Kalis *et al.* 1999; Rothkamp *et al.* 2009)	Study 2[ǂ]		5.6	5
		Study 3[ǂǂ]		8.2	5
Correa-Valencia *et al.* (2017)	Sedimentation-centrifugation-one-step HPC	Study 1	27 (P)	0	7.4
Noll *et al.* (2017)	Sedimentation-centrifugation-one-step HPC	Study 1	237 (I)	7.2	5.5

MAP, *Mycobacterium avium* subsp. *paratuberculosis*; BAC, benzalkonium chloride; NaOH, sodium hydroxide; OA, oxalic acid; HPC, hexadecylpyridinium chloride; NADC, National Animal Disease Center; FLI, Friedrich-Loeffler-Institut-Amtliche Methodensammlung (Official Collection of Methods). (http://www.fli.bund.de/fileadmin/dam_uploads/Publikationen/Amtliche_Methodensammlung/Methodensammlung_201204.pdf; http://www.fli.bund.de/fileadmin/dam_uploads/Publikationen/Methodensammlung_2010-07-07.pdf); NR, not reported.

[+] Cultured in pool (P) or individual (I)

[*] MAP-isolation rate not reported because it was a control of Study 1

[**] MAP-isolation rate not presented because it was a control of Study 3

[£] 1 g-sample

[ff] 2 g-sample

[‡] At cow-level

[‡‡] At herd level

[†] Low speed centrifugation (1,000×g for 15 min)

[††] High speed centrifugation (3,000×g for 15 min)

[ǂ] Cultured on Herrold's egg yolk medium (HEYM)

[ǂǂ] Culture on Lowenstein-Jensen medium

The MAP isolation rate from both bovine fecal and environmental samples ranged from 0 to 100% and the same for the contamination rate. The three main protocols, according to the number of fecal samples analyzed (n = 36,432) and the studies included (20/45), corresponded to 84.6% of the total samples included in this SR. These protocols are presented according to the number of fecal samples evaluated. The centrifugation-one-step HPC protocol was used for over 22,154 fecal samples from six studies. The average MAP isolation rate for this protocol was 3.99% (886/22,154), ranging from 1.64 to 5.7%; and the average contamination rate was 0.17% (38/22,154), ranging from 0.07 to 30%. The sedimentation-centrifugation-NaOH/OA protocol was used for over 12,830 fecal samples in nine studies. The average MAP isolation rate for this protocol was 8.57% (1,100/12,830), ranging from 3.3 to 78%, while the average contamination rate was 19.9% (2,554/12,830), ranging from 0 to 23%. Double-incubation-centrifugation-one-step HPC was used for over 1,448 fecal samples in five studies. The average MAP isolation rate for this protocol was 8.14% (118/1,448), ranging from 1.6 to 83.3%, while the average contamination rate was 17.4% (252/1,448), ranging from 0 to 21.5%. The most commonly used solid medium was Herrold's egg yolk medium (HEYM) (26/45).

From here and according to the decontamination protocols described by each paper, the frequency was as follows (from highest to lowest): the centrifugation-one-step HPC-water protocol, the double-incubation-centrifugation-one-step HPC-water protocol, and the sedimentation protocol were reported by four of the relevant articles each one; the sedimentation-one-step HPC-water protocol was reported by three of the relevant articles; the sedimentation-centrifugation-one-step HPC was reported by two of the relevant articles; finally, the centrifugation-one-step HPC-water protocol, the double centrifugation-one-step HPC protocol, the double-incubation-double centrifugation-one-step HPC-water protocol, the sedimentation-one-step BAC protocol, the sedimentation-one-step HPC protocol, the sedimentation-centrifugation-one-step HPC-water protocol, and the sedimentation-centrifugation- one-step NaOH/OA protocol were reported by one of the relevant articles each one. All previous results are based only on single data reports (i.e., individual, pool).

DISCUSSION

The aim of this SR was to collect, describe, and analyze studies reporting the effects of decontamination protocols on solid media culture results when complex matrices such as feces and environmental samples were processed for MAP detection, allowing us to answer the research question of how decontamination protocols affect the growth of microorganisms other than MAP-irrelevant microorganisms in solid media cultures in bovine fecal and environmental samples. This question is important because microbiological contaminants have been shown to inhibit the growth of MAP or to hide MAP colonies in solid media (Secott *et al*., 1999; Whittington, 2009). Thus, contaminants complicate, delay, and increase the cost of MAP culture.

Questions arising from this report are not intended to extend to liquid media, other than bovine samples, and other-than-fecal and environmental sample cultivation. The scope of our SR, defined at the protocol-definition stage, considered a solid culture of environmental and bovine fecal samples, since the technique and matrices are now considered routine procedures and are available from certain diagnostic laboratories. In addition, there is evidence from several laboratories that solid media are cheaper, less instrumentation is required, and identification of the organism is simpler (Whittington, 2010; Dane *et al*., 2022). Regardless of the analysis matrix, there is always a need to control the contamination and consequently the IM-related results, which apparently affect more liquid cultures compared to solid cultures (Whittington, 2009, 2010). Moreover, when the definition of the strain (MAP-type) is considered, it cannot be assumed that all strains of MAP have the same or similar antimicrobial resistance patterns, so each combination of antibiotics used both during decontamination procedures or within culture media (solid or liquid) will need to be carefully evaluated for each MAP type (Whittington, 2009).

Considering our methodology, the databases allowed access to information from 1910 to the present. Since the first report of consistent solid-media cultivation of MAP was published in 1912 (Twort *et al*., 1912), many variations of the original culture methods have been explored and implemented. Nevertheless, it should be noted that advances in the culture of MAP from fecal and environmental samples have been delayed by the distinctive growth characteristics of this bacterium. In addition, the need for a prolonged incubation period to culture the organism has led to numerous studies searching for a proper combination of decontaminants and antimicrobials that can effectively inhibit bacterial and fungal contaminants without affecting the growth of the primary isolate (Merkal & Curran, 1974; Jorgensen, 1982; Whitlock & Rosenberger, 1990; Stabel, 1997; Gwóźdź, 2006; Whittington, 2009).

Considering the relationship between the effectiveness of decontamination and the success of MAP isolation, we reported both contamination and MAP isolation rates. Both should be included in the methodological assessment of MAP-related studies (Stabel, 1997; Nielsen *et al*., 2004). Several reasons for the decontamination steps may explain the variability in the reported estimates (both contamination and MAP recovery rate) from solid cultures aimed at detecting MAP.

No differences related to sample quantity were reported or mentioned by any of the authors of the relevant studies. Nevertheless, the amount of matrix cultured (i.e.,

feces, environmental sources) should be standardized, as other researchers have reported to influence the contamination rate. Only to mention one case, Stabel (1997) recommended using 2 g of fecal samples rather than 1 g along with the Cornell decontamination method (double incubation-centrifugation) on HEYM culture to improve detection of subclinically MAP-infected animals.

Different methods for reducing bacterial and fungal contamination, including oxalic acid (OA), sodium hydroxide (NaOH), sodium hypochlorite, phenol, benzalkonium chloride (BAC), and hexadecylpyridinium chloride (HPC), have been evaluated for the isolation of MAP, emphasizing the latter two chemical decontaminants (Stabel, 1997). The cationic quaternary ammonium compound HPC was first used in the USA, replacing BAC (Merkal, 1984), and is now the basis for recent protocols in many countries, including North and South America, Europe, and Australasia. Eamens *et al.* (2000) concluded that longer double incubation times (24 and 48 h) in HPC and a mixture of amphotericin B, nalidixic acid, and vancomycin (VAN) were positively related to lower contamination. Mokresh *et al.* (1989) also found a reduction by about 2 log10 after exposure to 0.75% HPC for 18 h. However, other authors have reported no effect of HPC for up to 5 days (Whipple *et al.*, 1992). Progressive loss of viable organisms occurs with each step-in sedimentation and centrifugation protocol, as only part of the material from one step is taken forward to the next step. Protocols with NaOH or OA reduced the concentration of MAP from cattle by 1–2 logs in 4 h, and BAC reduced it by 1 log; HPC did not affect the viability of MAP over a 5-d incubation at concentrations of up to 1% (Jorgensen, 1982; Merkal *et al.*, 1982; Whipple & Merkal, 1983).

Different individuals and combinations of antibiotics have been studied to determine the concentration with maximal effect on contaminating microorganisms, yet with minimal influence on mycobacteria. Antibiotic combinations used include the commercially available PANTA™ Plus (Whittington *et al.*, 1998; Gumber & Whittington, 2007), a mixture of amphotericin B, nalidixic acid, and VAN (Reddacliff *et al.*, 2003; Whittington, 2009), or the less frequently used alone or a combination of penicillin, chloramphenicol, and amphotericin B (Jorgensen, 1982; Whipple *et al.*, 1992). Separately from their inhibitory effect on contaminating bacteria and fungi, these components can also have a negative effect on the growth of mycobacteria, which is strainand antibiotic concentration-dependent (Gumber & Whittington, 2007). Reddacliff *et al.* (2003) reported losses related to carryover effects of the VAN-based incubation (but not statistically significant) when analyzing sheep feces of 1.7 $\log_{10}$, due to 72 h incubation.

When Whipple *et al.* (1992) compared fecal culture techniques on solid media, authors found that conventional sedimentation procedures gave comparable results to those using centrifugation with double incubation and antibiotics (Cornell method) or centrifugation alone. In contrast, Whitlock & Rosenberger (1990) found an increased sensitivity of detection by 3-fold when comparing centrifugation-based to the sedimentation method.

Centrifugal concentration of bovine fecal specimens has been shown to shorten the incubation time required and improve analytical sensitivity (Reddacliff *et al.*, 2003). However, centrifugation did not increase the isolation rate of MAP from fecal specimens when compared to the standard sedimentation method (Kim *et al.*, 1989; Whitlock *et al.*, 1989), and, as for feces, this tends to increase the contamination rate (Reddacliff *et al.*, 2003).

According to Kim *et al.* (1989), double centrifugation-based protocols increase the chance of detecting animals shedding small numbers of MAP organisms. Nevertheless, the same authors reported a significant increase in contamination in centrifuged fecal samples compared to those processed by sedimentation in HEYM cultures (60 vs. 26%), with similar overall detection rates. On the other hand, Ridge (1993) reported a method for bovine fecal sample processing, involving the same protocol (double centrifugation), and then culturing on two stages-culture systems (liquid and 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 longer storage of the samples.

Whitlock & Rosenberger (1990) described an alternate centrifugation-double incubation method, with centrifugation speeds lower than the Cornell method, to reduce contamination problems. In their experience, centrifugation increased culture sensitivity by up to three-fold in cattle shedding low numbers of MAP compared with sedimentation. Eamens *et al.* (2000) results reinforce and extend several reports on concentration methods using centrifugation with or without double-incubation found to be more sensitive, compared to those based on sedimentation (from 39 to 68%), since Sweeney *et al.* (1996) found that laboratories employing centrifugation methods had increased detection of positive samples (19%) compared to those using sedimentation as decontamination protocol (15%).

A suggested step-by-step decontamination protocol to be used on bovine fecal samples for the cultivation of MAP in solid media, as defined for the purpose of this review, is presented in Figure 2 and defined according to the results of this SR. To make this decision, we decided to focus on fecal samples only as well as on the lowest contamination rate rather than the MAP recovery, as the latter will depend on other variables that are not directly related to the decontamination process (e.g., MAP viability and quantity in the original sample, elimination intermittency, disease phase, origin of samples, season, and environmental factors).

The centrifugation-one-step HPC seems to be a more reliable protocol for these matrices in terms of the number of samples that have been analyzed with this method (n = 22,154), the lowest contamination rate of the three main protocols presented previously (0.17 vs. 19.9 and

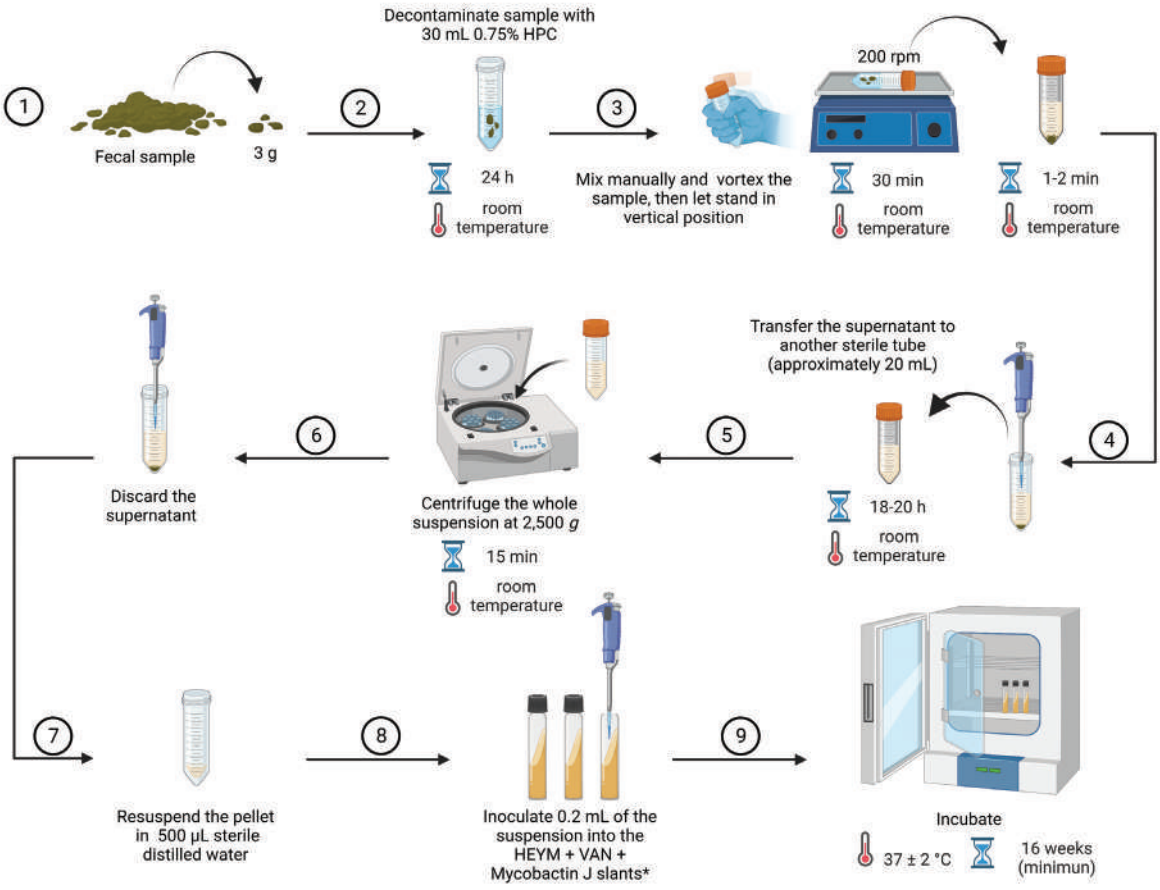

Figure 2.
Suggested decontamination procedure for solid media cultivation of *Mycobacterium avium* subsp. *paratuberculosis* in bovine fecal samples (centrifugation-one-step HPC). The steps were defined as follows: 1) sample quantity (g), 2) decontamination solution addition, 3) post-decontamination, 4) incubation, 5) post-incubation centrifugation (g), 6) supernatant handling, 7) pellet handling, 8) inoculation on solid culture media*, and 9) culture incubation.
rpm, revolutions per minute; HEYM, Herrold's Yolk Medium; HPC, hexadecylpyridinium chloride; VAN, amphotericin B, nalidixic acid and vancomycin mixture.
*Commercially available slopes from Becton, Dickinson and Company (HEYM + VAN + Mycobactin J).

17.4%), the "history" of the method (2011-2017), includes the HPC as the chemical decontaminant —recognized as the mainly used decontaminant nowadays, it is supported by the literature as a sensitive protocol in terms of MAP recovery and describes fewer steps to be followed, which translates to less time and instrumentation.

Does this information suggest a change? The answer is, definitively. The consistent use of nonstandard methodologies among studies in this SR makes pooling or comparisons problematic, even given the option of performing a meta-analysis because of the heterogeneity of the data (Dohoo *et al.*, 2014). The difficulty is that there is no true 'gold standard' protocol for MAP in bovine fecal and environmental samples. This problem could be partly addressed by the consistent use of a single reference protocol, thus allowing comparison of diagnostic accuracy across different studies.

A previous SR evaluating rapid tests for bacterial intestinal pathogens in food and feces also reported an overall limited quality of the included studies (Abubakar *et al.*, 2007). Other researchers have concluded that the conduct of SRs and meta-analyses for the evaluation of diagnostic tests have been hampered by the poor quality of reporting of diagnostic studies (Abubakar *et al.*, 2007; Page *et al.*, 2021). Whether similar deficiencies observed in this SR were caused by poor study design or poor reporting is unknown and should be further explored. Efforts have been made in recent years to encourage standardization of methods for reporting primary research and SRs via projects such as the Standards for Reporting Diagnostic Accuracy (STARD) initiative, the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement, and the development of the Quality Assessment of Diagnostic Accuracy Studies (QUADAS) tool, all

considered inside the Equators network (https://www.equator-network.org/). Even within the length restrictions imposed by publishing journals, the items listed within these tools must be addressed. Although these tools were developed for application in the human health field, researchers in the areas of veterinary and agri-food public health should be encouraged to adopt similar guidelines. The guidelines provided by these tools should also be considered during the design and conduct of studies on the diagnostic accuracy and related procedures.

The use of a wide array of decontamination protocols, which are compared against an equally wide display of reference protocols, makes it difficult to compare results from different studies. This challenge was highlighted in the current analysis. Results of different studies were found —in some way, contradictory, maybe because of the different matrices (including factors affecting their characteristics, as previously mentioned) and laboratory procedures and facilities. Therefore, there is still a need for further research on the standardization of the protocols, since there seems to be a lack of consistency in the method of isolating MAP from submitted samples at the laboratory level. This void directly affects reports worldwide, leading to incomparable, unrepeatable, and undiscussable results, as culture is still considered the gold standard test for PTB diagnosis (Gilardoni *et al*., 2012; Dane *et al*., 2022).

In addition, it is important to highlight the need for a better definition of the "contamination" outcomes. Contamination by IMs can reduce the diagnostic sensitivity of culture and increase the complexity and cost of confirming the presence of MAP (Whittington, 2009). The detection of mycobacteria other than MAP presents a disadvantage to the routine processing of samples because of the added cost of subculture into additional cultivation and PCR confirmation and a failure to obtain a result for MAP due to contamination. In this same sense, there is a lack of consistency in the MAP culture-related literature about the meaning of "contamination", which makes comparisons between different studies. Contamination can refer to a mixed culture of MAP and other-than MAP microorganisms, a light growth of irrelevant microorganisms or a complete overgrowth of the medium. The lack of case definition for "contamination" when culture outcomes are reported, hinders comparisons among studies.

Then, what other information or research is required on this topic? We suggest separating the research approaches to adapt an optimal decontamination protocol according to each matrix. The above suggested protocol (i.e., centrifugation-one-step HPC), used by four articles in the present SR (Fernández-Silva *et al*., 2011; Donat *et al*., 2015, 2016; Heuvelink *et al*., 2017) could be considered for further analysis since low contamination (almost "negligible") was reported. In addition, contamination-related results should always be reported along with MAP culture results to allow the reader to consider this information while interpreting the results.

Regarding the systematic process carried out per se herein, the authors agreed not to perform the study quality appraisal, as recommended by the PRISMA guidelines, since the answer to the question of interest in this SR is not usually reported as a primary finding. In our case, 27 articles meeting the inclusion criteria were evaluated, and only seven explicitly considered our research question as an objective. The other 20 articles described the decontamination protocol in the Materials and Methods section and reported their results on the contamination rate and MAP recovery in the results or discussion sections. In addition, available quality checklists are not applicable to our purpose, leading to negative results for the internal and external validity appraisal, when in fact they answered our question. Nevertheless, definitive articles incorporated into this SR were always obtained through a consensus on basic eligibility among the authors.

According to the analysis, the concept of contamination was responsible for the limited range of findings. Nevertheless, if we had not considered this, the initial work matrix would have included all the articles that reported solid media, in cattle, and in MAP, which included prevalence and incidence studies, control program evaluation or description, and longitudinal studies, among others, which were out of the purpose of the search. In conclusion, the authors consider the first search to be sufficiently restrictive based on the investigation question.

Our SR has strengths. We followed a written protocol based on a clearly stated and delimited research question. We performed a comprehensive literature search in general-purpose databases, search engines, journals, conference proceedings, book chapters, and books from 1910 to the present using pre-established and explicit inclusion/exclusion criteria. No geographical or temporal constraints were considered in this study. We recognize that our SR contains a modest sample of 27 original articles, given its antiquity in the culture of the microorganism of interest. However, our findings represent the most comprehensive summary of the effects of decontamination protocols on the outcome of solid media for MAP identification.

Our SR had some limitations. When results from snowballing were obtained, we found that the terms "contamina*" and "decontamina*" were the cause that three articles were not detected through the initial searching, being these terms found to be excluded in some specific cases. The grey literature has not been fully considered.

Considering the currently available data, it is difficult to systematically review the literature on this subject, as microbial overgrowth is frequently reported as a secondary outcome instead of a primary objective. The results of this SR demonstrate that there is considerable variability in the percentage of overgrown samples among studies because individual study designs and decontamination protocol characteristics vary considerably among reports.

In conclusion, this SR highlights the need for further refinement of decontamination protocols to minimize the

losses of viable MAP during the processing of bovine fecal and environmental samples because the compilation of information presented herein would orient to protocol improvements and to explore research approaches. We found that sample matrix and quantity, HPC amount, antibiotic concentration, and time-to-contact during incubation, incubation temperature, use of MAP concentration techniques (as filtration), as well as media used and its enrichment type and antibiotic mix, can explain (at some, but no well-established level) the variability in the cultures' outcomes (MAP detection and contamination rate). Finally, it seems that the centrifugation-one-step HPC protocol demonstrates the best results in terms of contamination rate. Nevertheless, it is important to consider the information with the prudence that it deserves and that the outcomes of interest (MAP recovery and contamination rate) may vary from case to case.

Future studies in this area of microbiology should follow standardized guidelines when designing and implementing studies and reporting their results, since the decontamination protocol is a key component in the sensitivity and specificity of the microbiological diagnosis of MAP, which is necessary in the definition of a true global prevalence of MAP.

Competing interests

The authors declare that they have no competing interest.

Author contributions

All the authors contributed to the conception and design of the study. N. M. C. conceived and suggested the design and methods of the SR. The literature search, data analysis, and critical revision of the manuscript were performed by all authors. The first draft of the manuscript was written by NMC, and all the authors commented on the previous versions of the manuscript. Figure 2 was designed by JMH. All authors have read and approved the final manuscript.

Data availability statement

Supplementary data (review protocol, template for data collection forms, and data extracted from included studies) are available upon request to the corresponding author, N.C.V. mariadelp.correa@udea.edu.co

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