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Comparative study of *Mycobacterium bovis* primary isolation methods

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ABSTRACT

For the definitive diagnosis of bovine tuberculosis, isolation of the etiologic agent is required. However, there is no consensus on the best methodology for isolation of *Mycobacterium bovis* in Brazil. This study evaluated the most used decontaminants and culture media in the country, in order to identify the best combination for the Brazilian samples. Three decontaminants – 2% sodium hydroxide (w/v), 0.75% hexadecylpiridinium chloride (w/v) and 5% sulphuric acid (v/v) and four culture media – 7H11 Middlebrook with additives and OADC supplement "A" (7H11 A), the same media with another supplement trademark (7H11 B), tuberculosis blood agar (B83) and Stonebrink's medium were compared. Regarding the isolation, there were no significant differences between the decontaminants and media combinations, except 7H11A combined to any decontaminant. However, the mean colonies score was significantly greater when the samples were decontaminated with 5% sulphuric acid and inoculated in 7H11 B or SB, without significant difference between them, although colonies appeared earlier on 7H11B than on SB. The trademark of OADC supplement influenced the isolation rate and the number of isolated colonies in Middlebrook 7H11. An incubation time of four weeks was required to detect all positive samples in 7H11 B after decontamination with 5% sulphuric acid but there was an increase in the number of colonies until the sixth week of incubation. Overall, the best strategy for the primary isolation of *M. bovis* from Brazilian samples was the decontamination with 5% sulphuric acid (final concentration) and inoculation in Middlebrook 7H11 medium formulated with OADC supplement "B".

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Introduction

Bovine tuberculosis is an important zoonotic disease caused by *Mycobacterium bovis*.[1,2] The isolation of the etiological agent is the definitive confirmatory diagnosis of the disease and is also important for epidemiological studies and for the validation of immunoassays.[1] However, the long time required for the isolation of the mycobacteria and the high level of tissue samples contamination are limiting factors. To facilitate the recovery of *M. bovis*, a range of pre-treatment (homogenization, decontamination and concentration) and use of an adequate culture medium are employed.[1,3]

The isolation and cultivation of *M. bovis* can only be carried out in a biosafety laboratory because it is a hazardous zoonotic agent. Some laboratories in the country perform this bacteriological diagnosis, but there is no consensus about the best decontaminant–medium combination to use for Brazilian samples.[4–7]

It is known that the type of decontaminant and the choice of media used affects the success of primary isolation.[8] The decontamination method traditionally used to isolate *M. bovis* from bovine tissues is Petroff method, which uses 4% sodium hydroxide (NaOH) solution.[9,10] However, previous studies showed a reduction of *M. bovis* viability in samples treated with 4% NaOH.[1,8,11,12] Hexadecylpyridinium chloride (HPC) and sulphuric acid (H_2SO_4) have been used as alternatives to Petroff method.[8,13] The decontamination with H_2SO_4 has been used in the Brazilian reference laboratory for animal diseases (LANAGRO) since 1985,[14] but few studies in Brazil have evaluated H_2SO_4 in comparison to the most used decontaminant methods.

Besides the decontaminants, the culture media also have an impact upon the sensitivity of *M. bovis* isolation. The genus *Mycobacterium* is highly demanding on nutrients, and it takes around five weeks to develop in a simple culture media like Stonebrink's medium.[3,16,17] The Middlebrook 7H11 medium, which is enriched with OADC supplement (oleic acid, albumin, dextrose and catalase), provides early isolation of *M. bovis*, reducing the incubation time to three weeks or less.[8,16,18,19] However, the higher concentration of nutrients and lower concentration of malachite green make Middlebrook 7H11 more susceptible to the growth of contaminants compared to Stonebrink's medium.[11,17] In addition, there are many reports of low quality OADC supplements commercially available, even responsible for bacillus growth inhibition.[20,21] The tuberculosis blood agar medium (B83) must be a good alternative for the primary isolation of *M. bovis* due to its strong selective ability, simple and low cost production.[9,22]

We have identified studies that evaluated the performance of some decontaminant or culture media for the isolation of *M. bovis*, but no previous study compared the effect of both decontaminant and culture media under the conditions of a Brazilian routine diagnostic laboratory. Therefore, the purpose of this work was to evaluate the decontamination and cultivation methods most used in Brazil, in order to identify the best combination to increase the growth of *M. bovis* on primary isolation and the number of infected samples identified, reduce the contamination and short the incubation period in conditions of a real routine diagnostic laboratory.

Materials and methods

Cattle samples

Seventy tissues fragment (lymph nodes and lungs), with lesions suggestive of tuberculosis, from seventy bovines condemned for tuberculosis during routine slaughterhouses inspection where used for this study. These samples were frozen and sent to the Brazilian reference laboratory for animal disease (LANAGRO) in Pedro Leopoldo, Minas Gerais where they were kept at −20 °C. Laboratory processing did not exceed 90 days post sample collection.

Preparation of tissues

Twenty grams of each lesion with the fat tissue removed, was cut in small pieces and macerated in 55 mL of 0.04% phenol red solution with the help of OMNI MIXER®, as a technique described by Robbe-Austerman et al.[23] The macerated tissue was filtrated in a double layer of cheesecloth[12] and the resultant filtrate of about 40% (m/v) was divided into four aliquots of 10 mL each.[1]

Decontamination

Each of the four 10 mL aliquots received one of the following treatments: the first aliquot was mixed to an equal volume of 10% H_2SO_4 (v/v) to obtain a final concentration of 5% acid according to MARKS.[13] The second aliquot was added to an equal volume of 4% NaOH (w/v) to a final concentration of 2%.[8] The third received an equivalent volume of 1.5% hexadecylpiridinium chloride (HPC) (w/v), to a final concentration of 0.75%.[12] The fourth aliquot was the control and received an equivalent volume of sterile distilled water (SDW). The four tubes were kept at room temperature for 30 min. After this period, the aliquots treated with H_2SO_4 and NaOH were neutralized with 20% NaOH and 10% H_2SO_4, respectively.

Inoculation of media

After the aforementioned protocols were performed, each one of the tissue suspensions was centrifuged at 3000 × *g* for 15 min, supernatant discarded and the pellet dissolved in 10 mL phosphate buffered saline. After 10 min of resting, 200 μL aliquot was inoculated in culture media slopes. For each treatment, two tubes of Stonebrink Leslie medium,[14] tuberculosis blood agar medium B83[9] and Middlebrook 7H11 agar with additives and OADC supplement Himedia™, here called 7H11 "A", and the same medium with OADC supplement Difco™, here called 7H11 "B",[23] were inoculated. The inoculated media were incubated at 36 °C ± 1 °C and examined weekly for the presence of colonies with the characteristics of *M. bovis*. All the slopes were incubated for 12 weeks.

The number of *M. bovis* colonies in each tube was recorded weekly and classified in scores according to Corner et al.[1] The time from the incubation to the appearance of the first identifiable growth of *M. bovis* was recorded too. Slopes in which contaminating organisms were considered to have interfered with the growth of *M. bovis* were considered contaminated.

Table 1 – Tissue homogenates (65 samples per group) were decontaminated with sulphuric acid (H_2SO_4), sodium hydroxide (NaOH), or hexadecylpiridinium chloride (HPC) and inoculated onto tuberculosis blood agar (B83), Middlebrook's 7H11 with additives and OADC supplement "B" (7H11 B), Middlebrook's 7H11 with additives and OADC supplement "A" (7H11 A) and Stonebrink's medium.

Decontaminants	H_2SO_4 5% (v/v)				NaOH 2% (w/v)				HPC 0.75% (w/v)			
Media	SB	7H11 A	7H11 B	B83	SB	7H11 A	7H11 B	B83	SB	7H11 A	7H11 B	B83
No. positive for *M. bovis*	62	55	62	61	61	52	61	60	60	47	58	58
Frequency of positive sample for *M. bovis* (% of total)	95.4[a]	84.6[bcd]	95.4[a]	93.8[a]	93.8[a]	80.0[bc]	93.8[a]	92.3[ad]	92.3[ad]	72.3[b]	89.2[ac]	89.2[ac]

Equal letters between two frequencies show no statistical differences ($p > 0.05$), according to the McNemar test.

Identification of M. bovis isolates

Representative colonies were identified as *M. bovis* by their colony characteristics and by real time polymerase chain reaction (RT-PCR).[24]

PCR reaction was prepared using the following reagents: 0.375 nM of each primer (Mbovis.88.F: 5′-CGC CTT CCT AAC CAG AAT TG-3′ and Mbovis.88.R: 5′-GGA GAG CGC CGT TGT AGG-3′), 10 μL of Fast EvaGreen qPCR Master Mix (Biotium, USA) in a 20 μL reaction. Thermocyler (QuantStudio7, Life Technologies, USA) was programmed as follows: 95 °C for 5 min, followed by 35 cycles at 95 °C for 15 s, 63 °C for 20 s, and 72 °C for 30 s, with the reading cycle length. The curve denaturation was performed at 72–99 °C, with intervals of fluorescence at every 1% rise in temperature.

On egg media the typical *M. bovis* colony was small, rounded, pale yellow to buff with irregular edges and granular surface. On agar medium they were white, thin, rough and flat with a central mound.[11]

Data analysis

For the analysis, the data from each of the 70 samples was divided into 12 subsamples, i.e., each subsample represented one of the 12 possible treatment/medium combination. The treatment combinations were compared on the basis of the infected samples detected, contaminated samples, number of *M. bovis* colonies that grew on the slopes and time to appearance of the first colonies. The frequency of positive samples detected and the frequency of slopes contamination were analyzed by Q Cochran test and McNemar test on a five percent significance level with the help of MedCalc™ program (free trial). Friedman and Dunn test on a five percent significance level was used to interpret the association between decontamination methods, media, and the first appearance time. These tests were also used to interpret the association between decontamination method, media and colony score using the Minitab™. Samples contaminated during incubation period were not consider in the analysis of the frequency of positive samples. For the first appearance time and colony score, it was considered just the samples in which *M. bovis* was isolated in at least one of the replicas of each type of culture medium.

Results and discussion

The aim of this study was to identify the best method for *M. bovis* primary isolation from bovine suggestive lesions. Various decontaminant/medium combinations were examined to find an optimum combination for routine diagnostic use in Brazilian samples. The combinations that identified the most positive samples were H_2SO_4–SB and H_2SO_4–7H11B (95.4%) (Table 1). However, this was not significantly more than those achieved with the other decontaminant–medium combinations, except 7H11 A combined to any decontaminant (McNemar test, $p < 0.05$). Our findings are consistent with previous observations: Corner et al.[1] also did not find significant variation in the number of positive samples on 7H11 medium compared to SB after decontamination with NaOH or HPC. It is possible that the effect of decontaminants and most of culture media on the frequency of positive samples was not apparent because of the high concentration of viable *M. bovis*, since we just used samples with suggestive lesions. This high concentration of the bacilli can be evidenced by the high mean colony score isolated by all combinations (Table 3). Significant differences between the treatments could have been found if we have used samples with low concentration of *M. bovis*. Medeiros et al., using paucibacilary bovine tissues, found differences in the number of positive samples according to the decontaminant used.[6]

The control samples (treated only with SDW) showed a high tissue contamination by other microorganisms (Table 2). It is possible that the procedure of sample collecting in slaughterhouses, associated with the storage conditions and the transit time to the laboratory, have contributed to the high contamination level found, confirming the need of decontamination.

When the samples were decontaminated with H_2SO_4, NaOH or HPC, the contamination level reduced dramatically, without significantly differences between the decontaminants and medium combinations. Evaluating the primary isolation of *M. bovis* from bovine lesions, Corner and Trajstman verified that 0.75% HPC was as efficient as 2% NaOH in controlling contaminants growth.[8] Holanda et al.[15] evaluating four decontamination methods – 0.75% HPC, 0.25% BC, 5% oxalic acid (OA) and 6%.

H_2SO_4 – also did not find any significant difference in decontamination efficiency. On the other hand, Ambrosio et al.[5] evaluating three decontamination methods – 0.75% HPC, 2% NaOH and 6% H_2SO_4 – found that 0.75% HPC was the most effective decontaminant. However, the samples used in that experiment were kept in saturated sodium borate solution, while those used in this study were at −20 °C. It is known that the degree of contamination and the kind of contaminant microorganisms varies considerably depending on the way the material is collected and stored.[12] Thus, it is possible that the

Table 2 – Tissue homogenates (79 samples per group) were decontaminated with sulphuric acid (H_2SO_4), sodium hydroxide (NaOH), or hexadecylpiridinium chloride (HPC) and inoculated onto tuberculosis blood agar (B83), Middlebrook's 7H11 with additives and OADC supplement "B" (7H11 B), Middlebrook's 7H11 with additives and OADC supplement "A" (7H11 A) and Stonebrink's medium.

Decontaminants	SDW				H_2SO_4 5% (v/v)				NaOH 2% (w/v)				NaOH 2% (w/v)			
Media	SB	7H11 A	7H11 B	B83	SB	7H11 A	7H11 B	B83	SB	7H11 A	7H11 B	B83	SB	7H11 A	7H11 B	B83
No. Contaminated samples	75	76	74	53	6	4	2	1	5	1	2	2	6	6	6	3
Frequency of contaminated samples (% of total)	94.9[a]	96.2[a]	93.7[a]	67.1[b]	7.6[c]	5.1[c]	2.5[c]	1.3[c]	6.3[c]	1.3[c]	2.5[c]	2.5[c]	7.6[c]	7.6[c]	7.6[c]	3.8[c]

Equal letters between two frequencies show no statistical differences ($p > 0.05$), according to the McNemar test.

variations in the sample storage mode have contributed to the divergent results observed.

Colonies appeared earlier in agar-based media (B83, 7H11 A and 7H11 B) than on the egg based medium (SB), irrespective of the decontaminant method (Table 3). Such difference has been reported previously, although all previous studies have stated the superiority of the egg based media in relation to the quantity of isolated colonies.[1,8,12] Because of these results, some authors suggest the use of two different types of culture media: one agar and another egg-based media.[1,18] However, in the present study, the mean colony score achieved with H_2SO_4–7H11B combination was the greatest and not significantly different from H_2SO_4–SB (Table 3). Thus, when 5% H_2SO_4 was used as a sample decontaminant, the agar Middlebrook 7H11 B medium was enough to obtain greater number of isolated colonies and earlier isolations, without requiring a second type of culture medium. Shorting the time of a bacteriological diagnosis is very important to take sanitary control decisions as early as possible to contain disease spread. The isolation of a larger quantity of colonies is significant for samples suspected of having small number of viable bacilli.

Most reagents used for sample decontamination have adverse effect on the growth of *M. bovis*, increasing the time of the first colonies appearance and reducing the number of recovered colonies.[1,11] Considering both time required for colonies appearance and number of recovered colonies, we could observe that the acid was the least toxic decontaminant for the mycobacteria (Table 3). Holanda et al.[15] also demonstrated lower toxicity of H_2SO_4, compared with HPC, benzalkonium chloride (BC) and OA. However, the authors did not compare it to NaOH. We identified only one study in Brazil that compared H_2SO_4, NaOH and HPC, concerning the contamination control of clinical specimens and toxicity for the *M. bovis*.[5] However, this study used samples preserved in sodium borate buffer, while the samples analyzed in the routine of the official laboratory of the Ministry of Agriculture are refrigerated or frozen.

On the other hand, there was an increase on incubation time required for colonies appearance in all the media evaluated in addition to reduction of the colonies score in 7H11 B and B83 media when samples were treated with HPC, what was a clear evidence of the greater toxicity of this decontaminant (Table 3). This toxic effect may lead to a false negative result if the clinical sample presents low amount of viable bacilli.[15] Previous studies have reported lower toxicity of HPC compared with NaOH. Corner and Trajtsman[8] observed fewer amounts of colonies on 7H11 and SB after sample decontamination with 2% NaOH compared to 0.75% HPC. Corner et al.[1] reported both a reduction of viable bacilli and colonies appearance delay when samples were previously decontaminated with NaOH 2%, compared with 0.75% HPC. Although none of these studies has tested the acid, the results for HPC and NaOH differs from our results. This discrepancy may be related to the metabolic state of *M. bovis* in tissues samples of each study. Different decontaminant may have disparate modes of action depending on the metabolic state of bacilli. Bacteria in

Table 3 – Tissue homogenates (39 per group) were inoculated onto tuberculosis blood agar (B83), Middlebrook's 7H11 with additives and OADC supplement "B" (7H11 B), Middlebrook's 7H11 with additives and OADC supplement "A" (7H11 A) and Stonebrink's media after being decontaminated with sulphuric acid (H_2SO_4), sodium hydroxide (NaOH), or hexadecylpiridinium chloride (HPC).

Decontaminants	H_2SO_4 5% (v/v)				NaOH 2% (w/v)				HPC 0.75% (w/v)			
Media	B83	7H11 A	7H11 B	SB	B83	7H11 A	7H11 B	SB	B83	7H11 A	7H11 B	SB
Mean colony scores	2.8[Ab]	2.7[Ab]	3.2[Aa]	3[ab]	2.6[Aa]	2[B]	2.7[a]	2.6[Ba]	2.1[ab]	1.9[Bb]	2.2[a]	2.4[Ba]
Mean time of first appearance (weeks)	3.4[Aa]	3.4[Aa]	2.9[Aa]	5.2[A]	3.5[Aa]	3.6[Aa]	2.9[Aa]	5.6[A]	4.2[b]	3.8[ab]	3.4[a]	5.8
Time require to obtain all positives (weeks)	06–08	04–06	0–4	08–10	06–08	04–06	0–4	08–10	10–12	06–08	06–08	08–10

Equal capital letters between two means of the same culture medium show no statistical differences between decontaminants. Equal lowercase letters between two means of culture media for each decontaminant group indicate no statistical differences between the media.

latency or active growth may be affected differently by each decontaminant used.[1]

It was shown that the trademark and probably the quality of OADC supplement influences the performance of Middlebrook 7H11 medium. The two 7H11 formulations were identical with the exception of the OADC supplement trademark. More sample were positive and more colonies were isolated in Middlebrook 7H11 formulated with OADC supplement "B" compared to the same medium when formulated with OADC Supplement "A" (Table 1). It is known that commercially available OADC supplements may not have the quality required to support proper growth of the mycobacteria.[25] Butler et al.[20] reported that some variation component between commercially available enrichment supplements could stimulate or inhibit the growth of the Mycobacteria. These data demonstrate the importance of previously testing a commercial trademark of the OADC supplement before using it in routine.

The period required to detect all positive samples vary according to decontaminant and culture medium combination (Table 3). For each decontaminant, the time for all samples become positive was longer on Stonebrink's medium compared to Middlebrook 7H11. Corner et al.[1] obtained the same result after samples decontaminated with HPC and BC, while no difference was observed when the samples were decontaminated with NaOH. HPC-B83 was the combination that resulted in greater period (10–12 weeks) for all samples to become positive. This can be explained by the difficult to identify the small growth colonies in this medium. Although Birn[22] has stressed the easy viewing of colonies in B83, this condition was not observed in this study, probably due to the presence of debris on the dark medium, which covered up the small colonies. Ikuta[26] had already reported the difficulty to identify colonies when using the B83 medium.

The incubation period necessary to achieve the highest level of sensitivity with the 7H11 medium was eight weeks. This period was reduced for four weeks when the samples were decontaminated with acid or base and inoculated in 7H11 medium with OADC supplement B. However, as the number of colonies grown until the sixth week of incubation (data not shown), it is recommended to incubate the samples treated with the acid and inoculated on 7H11 B for up to six weeks to ensure maximum isolation.

Our data supports the recommendation of Corner and Trajstman[8] that the combination of medium and decontaminant should be consider in the primary isolation of *M. bovis*. In summary, we have shown that the use of 5% H_2SO_4 (final concentration) with Middlebrook 7H11 formulated with OADC supplement "B" incubation for up to six weeks resulted in the greatest chance of successfully isolating *M. bovis* from bovine suggestive lesions in the shortest time possible. However, when resources are limited, it can be considered some criteria for choosing the decontaminant–medium combination. If the criterion of choice is number of positive samples detected, it is possible to opt for cheaper culture media as SB or B83 medium after samples decontamination with the H_2SO_4, NaOH or HPC. If a faster diagnosis is important, than it is necessary to opt for 7H11B medium after decontamination with H_2SO_4 or NaOH, which were the combinations that provided greater precocity of isolation. If the number of recovered colonies has some importance to the diagnosis, especially when a low concentration of bacilli in the sample is suspected, then the Middlebrook 7H11B medium after sample decontamination with 5% H_2SO_4 (final concentration) could be the choice.

Conflicts of interest

The authors declare no conflicts of interest.

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