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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% hexadecylpyridinium chloride (w/v) and 5% sulphuric acid (v/v) and four culture media – 7H11 Middlebrook with additives and oleic acid, albumin, dextrose and catalase supplement "A" (7H11 A), the same media with another supplement trademark (7H11 B), tuberculosis blood agar and Stonebrink's medium were compared. Regarding the isolation, there were no significant differences between the decontaminants–media combinations, except 7H11 A 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 7H11 B than on SB. The trademark of oleic acid, albumin, dextrose and catalase 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] Besides the public health impacts, the disease leads to economic losses mainly due to discarding of carcasses in slaughterhouses and restrictions to international trade of meat and live animals.

The bases of the National Program for Control and Eradication of Brucellosis and Tuberculosis (PNCEBT) in Brazil are the elimination of all reactive bovines to the tuberculin test. In addition, there is a surveillance in slaughterhouses under federal inspection. Therefore, all suggestive lesions detected during slaughter in establishments with Federal Inspection Service (SIF) are sent to an official laboratory for bacteriological confirmation. Thus, the farm can be traced back and the other animals can be tested.[2]

The isolation of the etiological agent is the definitive confirmatory diagnosis of the disease. This bacteriological diagnosis 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]

Some laboratories in the country perform the diagnosis of animal tuberculosis by isolation and identification of the agent, but there is no consensus about the best decontaminant – medium combination to use for Brazilian samples.[4–7]

The decontamination method traditionally used to isolate *M. bovis* from bovine tissues is Petroff method, which uses 4% sodium hydroxide (NaOH) solution.[8,9] The main problem of a decontaminant reagent is its adverse effects for *M. bovis* at the concentration that it is used to give a complete control of contamination.[1,11,12] Previous studies showed a reduction of *M. bovis* viability in samples treated with 4% NaOH.[1,11–13] Hexadecylpyridinium chloride (HPC) and sulphuric acid (H_2SO_4) have been used as alternatives to Petroff method.[11,14] The decontamination with sulphuric acid has been used in the Brazilian reference laboratory for animal diseases (LANAGRO) since 1985,[15] but few studies in Brazil have evaluated H_2SO_4 in comparison to the most used decontaminant methods. Holanda et al.[16] demonstrated lower toxicity of H_2SO_4, compared with HPC, benzalkonium chloride (BC) and oxalic acid (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.

Besides the decontamination methods, 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.[3,17,18] 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.[11,17,19,20] 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.[12,18] In addition, there are many reports of low quality OADC supplements commercially available, even responsible for bacillus growth inhibition.[21,22] 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.[8,23]

It is known that the type of decontaminant and the choice of media used affects the success of primary isolation and must be adjusted for the conditions in which the bacteriological diagnosis is performed.[11] In Brazilian routine laboratories, there is no consensus on the best methodology for primary isolation of *M. bovis*. 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 combination of the decontamination and cultivation methods most used in Brazil, in order to identify the best option to increase the diagnostic accuracy and reduce the required time for isolating of the *M. bovis* the microorganism 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.[24] The macerated tissue was filtrated in a double layer of cheesecloth[13] 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% sulphuric acid (H_2SO_4) (v/v) to obtain a final concentration of 5% acid according MARKS.[14] The second aliquot was added to an equal volume of 4% sodium hydroxide (NaOH) (w/v) to a final concentration of 2%.[11] The third received an equivalent volume of 1.5% hexadecylpyridinium chloride (HPC) (w/v), to a final concentration of 0.75%.[13] The fourth aliquot was

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