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Primary isolation of *Mycobacterium bovis* from bovine tissues: Conditions for maximising the number of positive cultures

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ABSTRACT

In studies of *Mycobacterium bovis* infection in animals a definitive diagnosis requires the isolation of the organism. However, the optimum conditions for the primary isolation of *M. bovis* have not been determined. The aim of this study was to determine for primary isolation of *M. bovis*, (a) the incubation time required to achieve maximum sensitivity (i.e., the number of positive samples identified), (b) the effect of decontaminants on bacterial growth rates, and (c) the influence of media and the number of slopes of media on the number of positive samples detected. Two agar-based media, modified Middlebrook 7H11 (7H11) and tuberculosis blood agar (B83), and an egg-based medium, Stonebrink's (SB) were compared. Three decontaminants, 2% (w/v) sodium hydroxide (NaOH), 0.75% (w/v) and 0.075% (w/v) cetylpyridinium chloride (CPC, also called hexadecylpyridinium chloride, HPC) and 0.5% (w/v) benzalkonium chloride (BC) were evaluated against treatment with sterile distilled water. The inoculated media slopes were incubated for up to 15 weeks. Colonies first appeared after 2 weeks on all media types and 75% of positive slopes were identified by 8 weeks. An incubation time of 15 weeks was required to identify all positive samples. The slowest growth was associated with inocula that contained the fewest viable bacilli. The time to the appearance of colonies was influenced by medium type: the median time to detection of colonies was 28 days on 7H11 and B83, and 36 days on SB. However, SB returned the greatest number of positive samples. Decontamination procedures increased the minimum incubation time required to detect positive cultures, probably due to the toxic effect of the decontaminants. Increasing the number of inoculated slopes resulted in an increased number of positive samples and a decreased time to the detection of colonies. Overall, the detection of *M. bovis* was significantly influenced by the choice of media, the decontaminant and the duration of incubation of cultures.

1. Introduction

Tuberculosis, caused by *Mycobacterium bovis*, is an infectious, chronic disease of domestic and wild animals, and the cause of zoonotic tuberculosis in humans. An accurate bacteriological diagnosis is often required when undertaking epidemiological and pathogenesis studies and for the validation of immunological assays. A definitive diagnosis can only be achieved by isolating *M. bovis* from clinical or post mortem specimens. In tuberculosis, there exists a wide range of infection states and the use of suboptimal procedures may lead to a bias in the detection of certain types of infection.

Members of the *Mycobacterium tuberculosis* complex, including *M. bovis*, grow slowly and compared with general bacteriological standards, require long incubation times, especially on primary isolation. Reference texts either do not recommend maximum incubation times

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(Murray et al., 2007), or recommend 8 weeks for primary isolation and suggest prolonged incubation (10–12 weeks or more) "in selected cases" (Kent and Kubica, 1985). The reported duration of incubation used for primary isolation of *M. bovis* from animal tissues ranges from 6 to 12 weeks (Miller et al., 2002; Hines et al., 2006; Collins et al., 1997; de Lisle et al., 2008; Costello et al., 1998; Schmitt et al., 1997; Drobniewski et al., 2003), but no explanations have been provided to support the times used.

Due to the difficulty in isolating *M. bovis* from clinical specimens, a range of pre-treatment and processing procedures (homogenisation, decontamination and concentration), and use of culture media that inhibit contaminating organisms, are employed to facilitate recovery of mycobacteria (Murray et al., 2007). Factors that impact on the success of primary culture are the types of decontaminants used, and the number and choice of media (Corner and Trajstman, 1988). Decontamination involves the use of toxic chemicals to which mycobacteria are generally more refractory than the contaminating microorganisms. Although their toxicity for mycobacteria varies, the chemicals generally decrease the number of viable bacilli in the specimens (Corner and Trajstman, 1988). However, it is not known if the decontamination process or the number of medium slopes has an influence on the optimum incubation time. The purpose of the current study was to examine factors that may influence the growth of *M. bovis* on primary isolation and the number of infected samples identified. The study allowed us to define the maximum incubation period required and the influence of decontaminants and shorter incubation times on the sensitivity of culture.

2. Materials and methods

2.1. Experimental design

The study was part of a bacteriology research project that supported the Australian Brucellosis and Tuberculosis Eradication program. Data was analysed from those samples where at least one inoculated slope was positive for *M. bovis* and where no slopes were lost due to contamination. This selection procedure ensured that the results were not biased against any medium that was more prone to contamination. The results of previous studies where we examined the proficiency of the four decontaminants, across a wide range of concentrations, to control contaminating micro-organisms and their toxicity for *M. bovis*, showed that there was considerable variation in the ability of different media to control contamination and the proficiency of different reagents to kill contaminants (Corner and Trajstman, 1988; Corner et al., 1995). It was also established that the media, in the absence of contamination, were all equally able to support the growth of a pure culture of *M. bovis* (Corner and Nicolacopoulos, 1988). Two studies were conducted (Table 1). Study 1 compared 4 treatments: sterile distilled water (SDW) and 3 decontaminants (cetylpyridinium chloride (CPC, Sigma–Aldrich Co.; also referred to as hexadecylpyridinium chloride or HPC in some publications, for example, Corner and Trajstman, 1988; McCallan et al., 2011), sodium hydroxide (NaOH) and benzalkonium chloride (BC, Sigma–Aldrich Co.), and 2 different medias, modified Middlebrook 7H11 agar medium without the addition of antibiotics (7H11)) and Stonebrink's egg-based medium (SB) prepared as per Gallagher and Horwill (1977) and Lesslie (1959) respectively. In Study 1, of the 81 samples examined, 30 were treated with the higher concentration of CPC, 51 with the lower concentration of CPC and only the latter 51 with BC. Study 2 compared 3 media (7H11, SB and tuberculosis blood agar (B83; Cousins et al., 1989)) after SDW or CPC decontamination. All media were prepared as slopes in 25 mL disposable plastic Universal tubes and each tube contained approximately 10 mL of medium. In Study 1, one slope of each medium was inoculated after each treatment. In Study 2, one slope was inoculated after SDW treatment and 3 slopes of each medium after decontamination. Only data for specimens where *M. bovis* was isolated on at least one slope was used in the analysis.

Table 1
Study designs showing the number of samples found positive for *M. bovis*, the treatment of samples and the culture media used.

Study	No. samples positive[a]	Treatments[b]	Media[c]
1	81	SDW NaOH 2% (w/v) CPC 0.75% (w/v) or 0.075% (w/v) BC 0.5% (w/v)	7H11 SB
2	303	SDW CPC 0.075% (w/v)	7H11 SB B83

[a] A positive sample was one where *M. bovis* was isolated from at least one inoculated slope of any of the medium–treatment combination and no slope was lost due to contamination. The total number of samples used in each study was not available.
[b] SDW – sterile distilled water; NaOH – sodium hydroxide; CPC – cetylpyridinium chloride; BC – benzalkonium chloride, and w/v (g/100 mL) was the final concentrations achieved in the tissue suspensions. In Study 1, 30 samples were treated with CPC 0.75% (w/v) and only 51 samples with BC.
[c] After all treatments in Study 1 and after SDW treatment in Study 2 one slope of each medium was inoculated, and after treatment with CPC in Study 2, three slopes of each medium were inoculated. 7H11 – modified Middlebrook's 7H11 agar medium; SB – Stonebrink's medium; and B83 – tuberculosis blood agar medium.

2.2. Cattle samples

The samples were collected from naturally infected cattle (*Bos taurus* and *Bos indicus* and *B. taurus* × *B. indicus* cross-bred beef breed) and experimentally infected cattle. Of the samples used in Study 1, 15 were obtained from cattle (Hereford-Shorthorn cross) experimentally infected with *M. bovis* strain M86/90 by the intratracheal route (Rothel et al., 1990). All the naturally infected animals had reacted to either the single intradermal tuberculin test, or were positive in an assay for bovine interferon-γ (Wood et al., 1991).

2.3. Sample collection

The tissue samples were collected aseptically at post mortem using sterile instruments to expose and collect tissue samples. All the samples were collected by the same