AN ALTERNATIVE BIPHASIC CULTURE SYSTEM FOR RECOVERY OF MYCOBACTERIA AND FOR DIFFERENTIATION OF SPECIES OTHER THAN M. TUBERCULOSIS COMPLEX FROM BLOOD SPECIMENS

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ABSTRACT

Mycobacteremia is an increasingly frequent complication of late-stage infection with the Human Immunodeficiency Virus (HIV). Several different procedures have been used to detect and characterize mycobacteria in blood. However, most of these are expensive and time consuming. Our study addressed the application of an alternative biphasic blood culture system containing modified Middlebrook 7H9 broth and Lowenstein Jensen medium (mod 7H9/LJ) developed for direct recovery of mycobacteria from blood. Additionally, we evaluated the possibility of rapid discrimination of Mycobacterium other than tubercle bacilli (MOTT) organisms by differential growth after simultaneous inoculation into control media and media altered by the addition of p-nitrobenzoic acid. In the first part of this study mod 7H9/LJ was compared with conventional 7H9/LJ. Mycobacterial growth curves were generated for M. tuberculosis and M. intracellulare in both control and test medium. In the second part of this study mycobacteria were recovered from 64 of 537 cultured specimens (11.9%). Growth was detected in 64 (100.0%) of the 7H9/LJ, and 62 (96.9%) of the mod 7H9/LJ biphasic bottles. The mean time to mycobacterial detection in the two systems was the same. For the third part of the study, a total of 1091 blood specimens were cultured in mod 7H9/LJ and in mod 7H9/LJ containing 500 µg/ml of p-nitrobenzoic acid. A total of 72% of all Mycobacterium avium complex (MAC) isolated from blood were presumptively identified correctly as MOTT within 27 days. This study indicates that the alternative biphasic culture system has great potential for use under laboratory conditions that prevail in developing countries.

Key words: Mycobacteria, blood, detection, mycobacteremia diagnosis

INTRODUCTION

Though historically it has been possible to recover M. tuberculosis from the blood of patients with miliary disease or during the initial dissemination phase of tuberculous infection, until the advent of the HIV pandemic, mycobacteremia was rarely detected (1,4).

The frequency of both silent and symptomatic mycobacteremia in Acquired Immunodeficiency Syndrome (AIDS) patients and the range of species...
encountered makes the culture of blood and bone marrow for mycobacteria an essential diagnostic tool for the modern laboratory (2,3,4,12).

The clinical demand for the diagnosis of disseminated mycobacterial disease in AIDS has led to the development of a number of commercial and non-commercial systems aimed at the detection of mycobacteria in sterile body fluids including lysis centrifugation, radiometric broth culture, and nucleic acid amplification and detection (1,10,16,17,20,21). Though these tools have proven sensitive and clinically useful, they are sometimes cumbersome or time-consuming and moreover, are too expensive to be used routinely by laboratories in the developing world. We sought to develop a simplified alternative method that would avoid practical problems associated with other systems and that would be easily affordable. This report describes the laboratory and clinical evaluation of a biphasic system containing modified Middlebrook 7H9 broth and Lowenstein-Jensen medium.

The study was carried out in three phases: 1) the laboratory evaluation of an alternative inexpensive enrichment method for Middlebrook 7H9 broth, 2) a clinical study comparing the sensitivity of detection of two biphasic culture systems, and 3) the evaluation of a method for direct species differentiation of mycobacteria growing from blood cultures.

**MATERIAL AND METHODS**

**Laboratory Evaluation of Modified Middlebrook Media.** Middlebrook 7H9 broth medium was enriched according to a standard protocol by the addition of ADC - bovine albumin fraction V, glucose and catalase (Difco Laboratories, Detroit, Michigan, USA) 10% by volume. A modified Middlebrook 7H9 broth was prepared by replacing the Middlebrook ADC enrichment with bovine serum (heat inactivated for 30 minutes at 56°C) to a final concentration of 10% and pH 7.2. The ability of the two medium preparations to support mycobacterial growth was compared by inoculation with pedigreed strains and serial quantitative subculture. Standard inocula of *M. tuberculosis* H37Rv (ATCC 25177) and *M. intracellulare* (ATCC 13950) were prepared in the following manner. Each strain was harvested in log-phase growth from 15 day cultures on Lowenstein-Jensen slants and placed in a tube containing 5 ml of Middlebrook 7H9 broth and glass beads. A homogenous suspension was prepared by shaking in a Vortex mixer for 3 minutes and the resulting suspension was allowed to sit while larger clumps settled to the bottom of the tube. The supernatant was then placed in a second tube and left undisturbed for 15 minutes to allow further settling of clumped organisms. The supernatant from this smooth suspension was then transferred to another tube and adjusted to a density equal to a 0.1 nm by the addition of 7H9 broth using a nephelometer. *M. tuberculosis* and *M. intracellulare* standardized inocula (0.5 ml each) were added to culture tubes containing 4.5 ml of standard or modified Middlebrook 7H9 broth. The capped tubes were incubated at 37°C and growth curves were constructed by performing quantitative culture of the test media on Lowenstein-Jensen slants at days 0, 3, 6, 9, and 12 after inoculation.

**Clinical Evaluation of Modified Middlebrook Media.** Biphasic culture systems were prepared in glass bottles of 50 ml capacity using Lowenstein-Jensen (LJ) medium for the solid phase and either standard or modified Middlebrook 7H9 broth for the liquid phase. The LJ medium was inspissated as an 8 ml slant and a 20 ml volume of enriched 7H9 broth was added that left roughly half of the LJ slant submerged and half exposed. The biphasic system using standard Middlebrook 7H9 broth with ADC (7H9/LJ) was used as control in a comparison with an identical system replacing the modified 7H9 broth described above (mod7H9/LJ). Both broth preparations were supplemented with 0.025% sodium polyanethol sulfonate to prevent coagulation, and antibiotics (20 μg/ml trimethoprim, 50 μg/ml carbenicillin, 10 μg/ml amphotericin B) to prevent contamination.

A total of 537 blood specimens were obtained from patients with AIDS with suspected disseminated mycobacterial infection hospitalized at the AIDS Training and Reference Center (CRTA) of São Paulo, Brazil, from January 1991 to July 1993. Ten milliliters of blood collected by venipuncture without anticoagulant after iodophor disinfection of the skin was divided into two 5 ml aliquots and immediately inoculated into the control (7H9) and test (mod7H9/LJ) media. The cultures were incubated upright at 37°C and observed daily for growth for up to 8 weeks. Bottles were inverted daily during the first week for reincultivation of the exposed solid media. Positive cultures were those demonstrating visible growth in either the solid or liquid medium, confirmed to be mycobacterial by Ziehl-Neelsen (Z-N) staining. Broth from the cultures remaining negative after 8
weeks of incubation was Z-N stained and examined for acid-fast bacilli (AFB) before being discarded to rule out growth not discovered by visual inspection.

Evaluation of a System for Direct Mycobacterial Species Differentiation. A total of 1091 blood specimens were obtained from AIDS patients with suspicion of disseminated mycobacterial infection hospitalized at the CRTA during the period from September 1993 to April 1995. Ten milliliters of blood collected by venipuncture without anticoagulant after iodophor disinfection of the skin was divided into two 5 ml aliquots for inoculation into each of two biphasic mod 7H9/LJ medium bottles prepared as detailed above. One of the bottles also contained 500 µg/ml of p-nitrobenzoic acid (PNB). The cultures were incubated upright at 37°C and inspected for growth daily for the first two weeks, at which time they were inverted for reinoculation of the exposed solid medium, and then weekly for an additional 6 weeks without further inversion. Bacterial growth detected by visual inspection was confirmed to be mycobacterial or contaminant on the basis of microscopic examination of stained preparations using the Z-N method. For all positive cultures, mycobacterial growth was compared in the paired bottles and cultures growing in medium containing PNB were presumptively classified as MOTT.

Identification of Mycobacterial Isolates. M. tuberculosis isolates were identified on the basis of p-nitrobenzoic acid susceptibility, 2-thiophenecarboxylic acid hydrazide resistance, niacin production, nitrate reduction and colony morphology. (5,11) MOTT were identified at the species level using standard methods.

Statistical Methods. The detection sensitivity of the two biphasic systems was compared using the Kappa agreement coefficient (8).

RESULTS

This study was carried out in three phases. In the first phase a modified Middlebrook 7H9 broth enrichment method was tested, replacing with bovine serum the more expensive Middlebrook ADC enrichment. Mycobacterial growth curves were generated for M. tuberculosis and M. intracellularare in both control and test medium by quantitative subculture at 3-day intervals. As shown in Fig. 1, similar logarithmic growth (7) was seen in both media.
In second phase of the study, 537 blood cultures were performed using a biphasic medium prepared with Lowenstein-Jensen and Middlebrook 7H9 broth. The cultures were performed in duplicate to compare mycobacterial recovery and contamination rates in bottles with standard ADC-enriched broth 7H9/LJ to those of bottles with the modified broth described above (mod 7H9/LJ). Mycobacteria were recovered from 64 of the 537 cultures (11.9%), 64 of which (100.0%) were detected by the 7H9/LJ bottles and 62 (96.9%) by the mod 7H9/LJ bottles. Kappa coefficient calculation showed excellent agreement (91.0%) between systems.

Of the 64 isolates, 37 (57.8%) were MAC and 24 (37.5%) were *M. tuberculosis* complex organisms. Three isolates could not be identified at the species level because of contaminating overgrowth or lack of growth on subculture. Primary contamination occurred in 11 (2.0%) and 8 (1.5%) of the 7H9/LJ and mod 7H9/LJ biphasic systems, respectively (Table 1).

The number of days required for detection of positive cultures did not differ between the two systems (Table 2). Mycobacterial growth was detected by inspection of both the liquid and solid phases of the culture system.

In the final phase of the study a method of species differentiation directly from the inoculated culture medium was evaluated. Pairs of biphasic 7H9/LJ bottles, one containing PNB, a specific inhibitor of *M. tuberculosis*, were inoculated with 5 ml of blood from AIDS patients with suspicion of disseminated mycobacterial infection. Of 1091 cultures, 58 (5.3%) were positive for mycobacteria. Of the 43 isolates given a final identification of MAC, 31 (72.1%) could be presumptively identified as MOTT over an average of 27 days on the basis of growth in the PNB-containing bottle. The 12 MAC isolates that failed to grow in the PNB-containing systems were all subsequently shown to be PNB-resistant. Misidentification of *M. tuberculosis* did not occur and all of these 15 isolates were growth inhibited by the selective biphasic bottle (Table 3).

Five isolates could not be identified at the species level because of either contaminating overgrowth or lack of growth on subculture. Primary contamination occurred in 12 (2.23%) biphasic systems.

**DISCUSSION**

The well-documented association of AIDS with Disseminated Mycobacterial Infection prompted the investigation of methods for the detection and identification of these organisms from blood specimens. Although biphasic blood cultures are commonly used for the isolation of bacteria and fungi, they are seldom used for the isolation of mycobacteria. The design of our study addressed the application of an alternative blood culture system for diagnosis and for monitoring the efficacy of therapy under the prevailing laboratory conditions in developing countries.

In this phase of our study, the simple replacement of ADC supplement with bovine serum to a final concentration of 10% in Middlebrook 7H9 medium did not affect the growth of *M. tuberculosis* or *M. intracellulare* (Fig. 1). This may be a useful substitution for containing costs (9), although, as with any other in-house media supplemented with serum or blood, factors may be present that possibly affect

### Table 1 - Recovery of mycobacteria and contamination rates from 537 blood specimens in two biphasic culture systems

<table>
<thead>
<tr>
<th>Biphasic system</th>
<th>Positive cultures n=64</th>
<th>Contaminated cultures n=12</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. avium</em> complex</td>
<td>64</td>
<td>12</td>
</tr>
<tr>
<td>MAC</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>MT</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>N1</td>
<td>32</td>
<td>23</td>
</tr>
</tbody>
</table>

*a Middlebrook 7H9 broth and Lowenstein Jensen  
b Modified Middlebrook 7H9 broth and Lowenstein Jensen  
MAC - Mycobacterium avium complex  
MT - Mycobacterium tuberculosis complex  
N1 - not identified species

### Table 2 - Number of days required for detection of the *M. tuberculosis* and *M. avium* complex organisms

<table>
<thead>
<tr>
<th>Organism</th>
<th>N° of isolates</th>
<th>7H9 / LJ</th>
<th>Mod 7H9 / LJ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Range</td>
<td>Mean</td>
</tr>
<tr>
<td><em>M. tuberculosis</em></td>
<td>23</td>
<td>18-60</td>
<td>31.3</td>
</tr>
<tr>
<td>MAC</td>
<td>32</td>
<td>14-60</td>
<td>27.1</td>
</tr>
</tbody>
</table>

*a Excluding 10 isolates that could not be identified  
b Mycobacterium avium complex
bacterial growth (14). Several methods have been used to recover mycobacteria from blood cultures. For example, Agy et al. (1), using the isolator system, recovered mycobacteria from 29 of 180 blood specimens submitted to culture (16.1%). Salfinger et al. (16) detected mycobacteria in 17.6% of the blood samples examined using sodium deoxycholate solution-lysis-centrifugation as the concentration technique, followed by inoculation of the sediments into radiometric BACTEC (7H13) (14). Strand et al. (18) reported that BACTEC 13A medium revealed the presence of mycobacteria in 63 of 1848 blood culture (3.4%). In the present study carried out for medium evaluation with clinical samples, mycobacteria were recovered from 64 of the 537 cultures (12.1%), 64 of which (100.0%) were detected with the 7H9/LJ bottles and 62 (96.9%) with the mod 7H9/LJ bottles. It should be noted also that our biphasic medium contained smaller amounts of liquid medium than usually recommended (3,6,11).

It is important to emphasize that factors outside the laboratory such as therapeutics and clinician indications for mycobacterial cultures may greatly influence the rate of recovery of mycobacteria from blood in these studies (1,4).

With respect to the time needed to detect mycobacterial growth, there was no significant difference between the biphasic systems studied. The wide range of growth detection times observed was probably due to the variable number of bacilli in the blood specimens. As demonstrated by Berlin et al. (3), the time of recovery is inversely proportional to the number of organisms inoculated.

The ability of mycobacteria to grow on media containing differentially inhibitory substances has repeatedly been shown to be of value for identification purposes (13,19).

The *M. tuberculosis* complex is inhibited by PNB at a concentration of 500 g/ml, whereas MOTT are resistant to this concentration. The latter, however, may present variation in sensitivity, with the existence of a small percentage of strains sensitive to certain drug concentrations, as proposed by Rastogi et al. (15) and Tsukamura et al. (19). In the present study, after a mean growth of 27 days in medium containing PNB, 31 strains (72.1%) were considered to be MOTT. This means that the laboratory can supply a presumptive MOTT result on the basis of the growth of these strains on medium containing PNB. All of these strains were later confirmed to be MAC on the basis of the results of biochemical tests.

With respect to cultures presenting growth only on control medium, i.e., strains sensitive to PNB, it was not possible to provide a presumptive result since 15 (100%) were identified as *M. tuberculosis* and 12 (27.9%) as MAC. One of the tests used for MOTT identification is resistance of the strain to 500 g/ml PNB. Resistance to this PNB concentration was observed in 12 MAC strains during their identification, suggesting that the bacillary load inoculated into the two flasks (control medium and medium mod. 7J9/LJ with PNB) was so low that it was inhibited by this drug concentration.

Two strains that presented growth in PNB-containing medium did not grow on control medium (with no PNB), suggesting that the samples were paucibacillary, permitting the occurrence of a difference between the inocula added to the two media.

For analysis of the agreement between methods for the presumptive identification of MOTT by growth on medium 7H9/LJ containing PNB and by MAC identification using biochemical methods, the Kappa coefficient was calculated with a 95% confidence interval, giving a value of 0.799 (0.696-0.901). This value may be considered a measure of the relative efficacy of the method of presumptive identification since it anticipates the definitive results obtained by the biochemical tests with reasonable concordance.

The differentiating ability of PNB-containing biphasic media appears to be sufficiently rapid and specific to be useful in a clinical laboratory, since identification of mycobacteria by conventional methods may take up to 1 month.

In conclusion, the alternative biphasic system is compact, self-contained, simple to use and
inexpensive. All of these advantages make it suitable for use under the prevailing laboratory conditions of developing countries.

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RESUMO

Sistema alternativo bifásico de cultura para o isolamento de micobactérie e triagem de espécies outras que não as do complexo *M.tuberculosis* a partir de espécimes de sangue

Micobacteremia constitui uma das manifestações oportunistas mais frequentes dos estágios avançados da infeção pelo vírus da imunodeficiência humana. Diversas metodologias têm sido propostas para o seu diagnóstico, no entanto, a maioria destas apresentam custo elevado ou complexidade de operação técnica. Baseados nestes fatos nos propusemos a avaliar um sistema alternativo de cultura bifásico contendo Middlebrook 7H9 modificado Lowenstein Jensen e bem adicional de ácido p-nitrobenzoico aos meios (mod 7H9/LJ+PNB). Para a primeira etapa do estudo comparou-se os meios mod 7H9/LJ e 7H9/LJ convencional, realizando-se a curva de crescimento das espécies *M. tuberculosis* e *M. intracellulare* em ambos os meios. Na segunda etapa do estudo isolou-se um total de 64 cepas de micobactérias a partir de 537 espécimes de sangue (11,9%), das quais 64 (100,0%) em 7H9/LJ e 62 (96,9%) em mod 7H9/LJ. Verificou-se um tempo semelhante de detecção destas micobactérias em ambos os sistemas. Para a terceira etapa do estudo, cultivou-se um total de 1091 espécimes de sangue nos sistemas mod 7H9/LJ e 7H9 mod/LJ contendo 500 μg/ml de ácido p-nitrobenzoico, constatando-se que um total de 72% dos organismos isolados e pertencentes ao complexo *Mycobacterium avium* puderam ser presuntivamente identificados como MOTT em 27 dias. Estes resultados, associados a simplicidade e baixo custo destes sistemas bifásicos constituem elementos potenciais para sua aplicabilidade na rotina diagnóstica em países em desenvolvimento.

Palavras-chave: Micobactérie, sangue, detecção, diagnóstico, micobacteremia

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