Detection of Mycobacteria by Radiometric and Standard Plate Procedures

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A group of 89 smear-positive sputum specimens were evaluated by radiometric and standard plate procedures to determine the methodology which would provide the earliest detection of mycobacteria and maximum test sensitivity. Digested non-decontaminated specimens were concentrated and inoculated into modified selective BACTEC radiometric 7H12 broth and Mitchison selective 7H10 agar. Sodium hydroxide (1.5% final concentration) was then used to decontaminate these specimens. They were then concentrated and inoculated into both selective and nonselective 7H12 radiometric broths and into selective 7H10 and nonselective Middlebrook 7H11 agar media. The specimen processing and media combinations providing the earliest detection were non-decontaminated specimens with modified selective 7H12 BACTEC broth and decontaminated specimens with 7H12 BACTEC broths. Maximum sensitivity (percent positive) was obtained by using non-decontaminated specimens on Mitchison selective 7H10 Agar (98%) or decontaminated specimens in 7H12 BACTEC broth (95%). The decontamination process was found to reduce significantly the number of mycobacteria in clinical specimens, particularly the mycobacteria other than Mycobacterium tuberculosis. The specimen processing-media combinations providing the earliest detection and maximum recovery of mycobacteria (100%) were non-decontaminated specimens with modified selective 7H12 BACTEC broth or Mitchison selective agar and decontaminated specimens with 7H12 BACTEC broth or 7H11 agar.

Rapid detection and drug susceptibility tests of mycobacterial isolates have been achieved with radiometric methods which use BACTEC instrumentation (Johnston Laboratories, Cockeysville, Md.) (2, 3, 4). Since many laboratories currently possess this instrumentation, it is possible to improve mycobacteria diagnostic services without major organizational changes. However, although the basic methodology for the radiometric detection of mycobacteria has been elucidated, there are few data available regarding the manner in which this system should be implemented to obtain the earliest detection while simultaneously achieving maximum recovery of mycobacteria. Therefore, the effects of the decontamination process, isolation media, and media combinations were evaluated.

MATERIALS AND METHODS

Eighty-nine sputum specimens, positive for acid-fast bacilli by fluorescent auramine staining (FAS) (1, 6), were obtained from patients treated for mycobacterial infections at Fitzsimons Army Medical Center, Aurora, Colo., or National Jewish Hospital and Research Center, Denver, Colo. All organisms were identified by means of currently recommended methods (1, 6, 8), and when appropriate, Schaeffer agglutination studies were performed (7).

The specimens were processed as outlined in Fig. 1 by using currently recommended methods (1, 7, 9); however, Sputolysin (Calbiochem-Behring, La Jolla, Calif.) was used as the mucolytic agent, centrifugation was performed at 2,400 × g for 30 min, and the specimens underwent concentration procedures both before and after decontamination with NaOH. After treatment with Sputolysin, the specimens were again centrifuged at 2,400 × g for 30 min. The digested/non-decontaminated sediment was then suspended in 4.5 ml of sterile distilled water and inoculated to the slide and media indicated in Fig. 1. The remaining portion of the sediment was then subjected to standard decontamination procedures by using 1.5% NaOH (final concentration), and after processing, they were inoculated into the prescribed media.

For the FAS procedure, 1 drop each of the non-decontaminated and the decontaminated suspensions was placed on glass slides. The number of organisms present were graded as follows: rare, 1 to 9 bacilli per 100 high-power fields (HPF); few, 1 to 9 bacilli per 10 HPF; moderate, 1 to 9 bacilli per HPF; and numerous,
DETECTION OF MYCOBACTERIA

RAW SPUTUM SPECIMEN

DIGESTED WITH 1:1 DILUTION SPYUTOLYSIN

DIGESTED/NON-DECONTAMINATED SPECIMEN

SLIDE AURAMINE FLUORESCENT STAIN

0.1 ml ND/MS-7H12 Broth

0.3 ml ND/S-7H10 Agar

STANDARD DECONTAMINATION OF SPECIMEN WITH 1.5% NaOH

DIGESTED/DECONTAMINATED SPECIMEN

SLIDE AURAMINE FLUORESCENT STAIN

0.2 ml D/S-7H12 Broth

0.3 ml D/7H10 Agar

0.2 ml D/7H12 Broth

0.3 ml D/7H11 Agar

FIG. 1. Mycobacteria specimen processing. Abbreviations: ND, non-decontaminated and digested specimen; MS-7H12, modified selective 7H12 BACTEC broth; S-7H12, selective 7H12 BACTEC broth; 7H12, nonselective 7H12 BACTEC broth; S-7H10, Mitchison selective 7H10 agar; 7H11, Middlebrook 7H11 agar; D, decontaminated and digested specimen.

>9 bacilli per HPF. A 0.1-ml volume of the non-decontaminated specimen was inoculated into the modified selective Middlebrook 7H12 radiometric broth (Johnston Laboratories) containing 5 μg of amphotericin B, 62.5 μg of polymyxin B, 50 μg of carbenicillin, and 5 μg of trimethoprim per ml. In addition, 0.3 ml was inoculated on Mitchison selective 7H10 agar (5). After decontamination and concentration processing, 0.2 ml was injected into Middlebrook selective 7H12 radiometric broth containing 5 μg of

FIG. 2. Detection times for 76 slowly growing mycobacteria by using BACTEC broths and plate media. Abbreviations ND/MS-7H12, non-decontaminated specimens with modified selective BACTEC broth; D/S-7H12, decontaminated specimens with selective BACTEC broth; D/7H12, decontaminated specimens with BACTEC broth; ND/S-7H10, non-decontaminated specimens with selective Mitchison 7H10 agar; D/S-7H10, decontaminated specimens with selective Mitchison 7H10 agar; D/7H11, decontaminated specimens with Middlebrook 7H11 agar. Numbers in parentheses indicate the actual percent positive for that point.
amphotericin B, 50 μg of polymyxin B, 25 μg of carbenicillin, and 2.5 μg of trimethoprim per ml and a nonselective Middlebrook 7H12 broth. In conjunction with the radiometric broths, routine mycobacteriological plate media were also inoculated, and 0.3 ml was inoculated onto nonselective Middlebrook 7H11 (6) and Mitchison selective 7H10 agars.

All radiometric bottles were flushed with a 5% CO₂–95% air mixture, and the plate media were sealed in polyethylene plastic bags before incubation at 37°C with 10% CO₂. Plate media were examined microscopically and macroscopically at the recommended intervals of 1, 2, 3, 4, 6, and 8 weeks. All negative plates were discarded after 8 weeks of incubation. Colony counts were performed on all positive plates, and isolates were identified by standard biochemical procedures (1, 6, 8). BACTEC 7H12 broths were read at days 1 through 7 and at 2, 3, 4, 6, and 8 weeks with a BACTEC 301, and the growth indices were recorded. The GI results are related directly to microbial metabolic activity; 100 U are equivalent to 0.025 μCi of liberated ¹⁴CO₂. Radiometric broths were considered positive if the difference between growth index of the negative control and the growth index of the test bottle was 6 or more growth index units. Except for the Mycobacterium fortuitum isolates, verification of mycobacteria was obtained by positive FAS slides and negative subculture on a 5% sheep blood agar plate. The Mycobacterium fortuitum isolates which grew on the blood agar plates were distinguished by their characteristic growth and positive FAS. After 8 weeks of incubation, all negative radiometric broths were examined by FAS and by subculture on 5% sheep blood agar. Contaminating organisms isolated from either radiometric broths or plate media were identified by currently recommended procedures (6). Antimicrobial minimal inhibitory concentration testing with Micro-Bio-Gram plates (Micro-Bio-Gram, Denver, Colo.) was also performed only on contaminants.

RESULTS

There were 89 FAS-positive clinical specimens included in the study; however, from both the radiometric and plate test systems, two specimens were contaminated and three failed to demonstrate growth. From the remaining samples, 51 M. tuberculosis, 6 Mycobacterium intracellulare, 6 Mycobacterium avium-M. intracellulare complexes, 5 M. avium, 8 Mycobacterium kansasii, and 8 M. fortuitum were isolated. Figure 2 presents the detection time data for the
slowly growing mycobacteria. After 7 days of incubation, the radiometric broth combinations providing the highest detection rate were non-decontaminated specimens in modified selective 7H12 BACTEC broth (78%) and decontaminated specimens in 7H12 BACTEC broth (68%), whereas for the same period, the plate providing the earliest detection rate was non-decontaminated specimens on selective 7H10 agar. Figure 3 presents the detection time data for eight isolates of rapidly growing M. fortuitum. After 72 h of incubation, the non-decontaminated specimens in 7H12 BACTEC broths detected 88% of the M. fortuitum specimens, whereas the remaining radiometric broths detected all the M. fortuitum. After 7 days, the plate media produced results comparable to those observed with the radiometric broths. Table 1 summarizes the sensitivity (number of positive specimens) for the various species processing-media combinations. The radiometric broth and nonradiometric medium providing the highest recovery rate were non-decontaminated specimens on selective 7H10 agar (98%) and decontaminated specimens with 7H12 BACTEC broth (95%). The lowest recovery rate was obtained when decontaminated specimens were used with selective 7H10 agar (83%). Non-decontaminated specimens inoculated into radiometric broths resulted in a 10% contamination rate. Identification of the 29 contaminants encountered in the study indicated that there were 9 Enterobacter cloacae; 6 Pseudomonas maltophilia; 3 Klebsiella pneumoniae; 3 Serratia marcescens; 2 each of Bacillus sp. and Staphylococcus epidermidis; and 1 each of Streptococcus faecalis, Streptococcus viridans, Aspergillus fumigatus, and Pseudomonas stutzeri. The minimal inhibitory concentration drug susceptibility test data revealed that 98% of the contaminants isolated were sensitive to one or more of the drug concentrations present in the selective media.

Figure 4 depicts the effects of the decontamination process and isolation media upon the number of mycobacteria recovered from the clinical specimens. Before decontamination with NaOH (Fig. 4a), 56% of the M. tuberculosis specimens produced colony counts greater than 200, whereas after decontamination counts greater than 200 were significantly fewer. This was particularly evident when decontaminated specimens were inoculated on selective 7H10 agar (33%). In addition, the percentage of specimens with counts of less than 25 increased from 8% to 35%. For specimens containing mycobacteria other than M. tuberculosis (MOTT bacilli, Fig. 4b), similar but even more dramatic reductions in plate counts were noted. Decontamination processing also affected FAS results (Table 2). Before decontamination, 66.7% of the specimens demonstrated numerous FAS results, but after decontamination, only 35.6% produced these data. Only 1.2% of the specimens were negative before decontamination but 16.6% were negative after decontamination processing. The effect of decontamination processing was evident for both M. tuberculosis and MOTT bacilli, but the MOTT bacilli were again affected more severely by this process. Before decontamination, the MOTT bacilli-containing specimens had no FAS negative results, and 19 of 33 specimens had numerous orga-
Figure 4 presents the data obtained when various combinations of specimens and media were used to detect slowly growing mycobacteria. The use of non-decontaminated specimens with modified selective 7H12 BACTEC broth combined with any other medium resulted in the earliest detection of mycobacteria. The use of non-decontaminated specimens on selective 7H10 agar in conjunction with any other medium provided the greatest percentage of positive specimens. The specimen processing-media combination providing complete recovery of test organisms and early detection consisted of non-decontaminated specimens with selective 7H10 agar and decontaminated specimens with 7H12 BACTEC broth. The lowest test sensitivity and longest detection times were observed when radiometric broths were omitted and decontaminated specimens were used with selective media.

Figure 6 reviews the test data for specimen-media combinations used to recover the rapidly growing mycobacteria. A variety of broth and plate media combinations resulted in maximum recovery and very early detection. Except when non-decontaminated specimens were used with modified selective 7H12 BACTEC broth, the radiometric broths detected all rapidly growing mycobacteria by 72 h. The plate media also provided excellent recovery but required 7 days of incubation before maximum recovery was achieved. Once again, the exclusion of radiometric broths and the use of decontaminated specimens with selective media resulted in delayed detection and reduced test sensitivity.
**DIscussion**

The methodology presented in Fig. 1 was used because plate media can support a greater test inoculum. The BACTEC radiometric broths were limited by their smaller media volume, and less inocula were required. In addition, preliminary studies revealed that the use of greater than 0.1 ml of non-decontaminated inocula with BACTEC broths resulted in high levels of contamination. The use of smaller volumes of inocula with the various radiometric broths did not appear to reduce significantly the ability of these media to provide early detection of mycobacteria.

The media presented in Fig. 2 and 3 were evaluated at different time intervals based upon currently accepted practice. Routinely, plate media are read weekly, whereas the potentially more sensitive radiometric broths required daily examination. Although this procedure may have delayed the detection of the *M. fortuitum*, after 72 h of incubation the 7H12 BACTEC broths had detected all of the isolates while the plate media detected only 75% after 7 days incubation. Since the vast majority of mycobacteria require considerably longer incubation periods than *M. fortuitum*, daily reading of plate media would not significantly improve detection, and this practice would be costly in terms of both time and money. Daily reading of radiometric broths, however, can be facilitated by automated BACTEC instrumentation.

The data presented in Fig. 2 and 3 revealed that the media providing the earliest detection of the slowly and rapidly growing mycobacteria were the modified selective and nonselective 7H12 BACTEC broths. With the heavy smear-positive specimens used in the study, the radiometric broths had a detection rate after 7 days of incubation of approximately twice that of the plate media. For slowly growing mycobacteria, the use of decontaminated specimens with selective media markedly prolonged detection times and provided fewer total positive results. This observation was not evident for the *M. fortuitum* specimens, but this might be attributed to the smaller number of specimens in this group and the high number of organisms present in the specimens. It also should be noted that although the use of radiometric methods resulted in 65 to 78% positive specimens by 7 days (Fig. 2), the sensitivity of the various plate media continuously improved so that by 3 weeks the use of routine media resulted in more positive specimens. The test sensitivities of the various media are presented in Table 1.

The plate count data found in Fig. 4 correlated with the observations in Table 2. The use of selective 7H10 agar with decontaminated specimens containing *M. tuberculosis* clearly resulted in lower plate counts (Fig. 4a). For the MOTT bacilli (Fig. 4b), this shift after decontamination was even more significant. Before decontamination, only 6% of the specimens had plate counts of less than 25 colonies, and 58% produced counts greater than 200; however, after decontamination, the plate counts with less than 25 colonies increased severalfold with a concurrent decrease in plate counts greater than 200. Decontaminated specimens inoculated on selective 7H10 agar demonstrated the greatest reduction of bacterial colonies. Although the study did include 84 positive specimens, no single medium recovered all organisms; this accounts for the variation in the number of positive specimens found in Fig. 4.

The sensitivity and detection capabilities of the various combinations of plate and broth media are presented in Fig. 5 and 6. For the slowly growing mycobacteria (Fig. 5), the use of non-decontaminated specimens with selective 7H10 agar and decontaminated specimens with 7H12 BACTEC broth provided maximum test sensitivity (100%) and very early detection of mycobacteria (67% after 7 days incubation).

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**Table 2. Effect of specimen decontamination on FAS staining results**

<table>
<thead>
<tr>
<th>Specimens</th>
<th>No.</th>
<th>Non-decontaminated</th>
<th>Decontaminated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Nar.</td>
<td>Rare</td>
</tr>
<tr>
<td><em>M. tuberculosis</em></td>
<td>51</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Mycobacteria other than <em>M. tuberculosis</em></td>
<td>33</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Total no. positive (% of total)</td>
<td>84</td>
<td>1 (1.2)</td>
<td>8 (9.5)</td>
</tr>
</tbody>
</table>

* Grouped as described in the text. Rare, 1 to 9 bacilli per 100 HPF (×450); few, 1 to 9 bacilli per 10 HPF; moderate, 1 to 9 bacilli per HPF; numerous, >9 bacilli per HPF.
FIG 5. Effect of media combinations on test sensitivity and detection times for slowly growing mycobacteria. The rightmost square in each row represents data only for single plate or broth medium. Abbreviations: ND/MS-7H12, non-decontaminated specimens with modified selective BACTEC broth; D/S-7H12, decontaminated specimens with selective BACTEC broth; D/7H12, decontaminated specimens with BACTEC broth; ND/S-7H10, non-decontaminated specimens with selective Mitchison 7H10 agar; D/S-7H10, decontaminated specimens with selective Mitchison 7H10 agar; D/7H11, decontaminated specimens with Middlebrook 7H11 agar.

These specimen processing-media combinations also provided excellent recovery and early detection of the rapidly growing mycobacteria; however, a number of other specimen processing-media combinations also provided good results. Since the contamination of broth medium often results in the failure to recover mycobacteria from clinical material, it is highly advisable to include an agar medium as part of the test system. The use of non-decontaminated specimens with selective 7H10 agar is recommended because its use resulted in the highest number of positive specimens of any single medium tested.

The use of only FAS-positive specimens was warranted by the results obtained from preliminary studies. These studies evaluated all clinical specimens submitted during a 1-month period. These specimens were processed consecutively.

FIG 6. Effect of media combinations on test sensitivity and detection times for rapidly growing mycobacteria. The rightmost square in each row represents data only for single plate or broth medium. Abbreviations: ND/MS-7H12, non-decontaminated specimens with modified selective BACTEC broth; D/S-7H12, decontaminated specimens with selective BACTEC broth; D/7H12, decontaminated specimens with BACTEC broth; ND/S-7H10, non-decontaminated specimens with selective Mitchison 7H10 agar; D/S-7H10, decontaminated specimens with selective Mitchison 7H10 agar; D/7H11, decontaminated specimens with Middlebrook 7H11 agar.
by the previously described methodology, and all 110 specimens were both smear and culture negative. Due to the financial and workload constraints, it was not feasible to continue studying this type of specimen. As a result, only FAS-positive specimens were evaluated.

It is imperative when processing specimens containing mycobacteria that the decontamination procedure be controlled carefully to avoid the loss of significant numbers of mycobacteria. This problem appears particularly serious in the recovery of MOTT bacilli. If a specimen is found to be highly contaminated, it is advisable to obtain a fresh specimen rather than subjecting the sample to extensive decontamination, which may render any mycobacteria present nonviable. If it is necessary to process such specimens, it would be advisable to include one or more nonselective Middlebrook series Tween broths to aid in the recovery of impaired mycobacteria.

The BACTEC radiometric system has proven to be a significant advancement for a variety of routine clinical microbiological procedures. The emerging BACTEC mycobacteria methodology also appears to have similar promise. This system has demonstrated the ability to rapidly detect mycobacteria in clinical specimens, and when employed in conjunction with the appropriate plate media, maximum recovery of mycobacteria may be anticipated.

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LITERATURE CITED