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Comparative Evaluation of Three Culture Methods for the Isolation of Mycobacteria from Clinical Samples

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We assessed the capacity of two liquid-medium culture methods with automated incubation and reading systems (MB/BacT ALERT 3D System and BACTEC MGIT 960 System) and one solid-medium culture method (Löwenstein–Jensen) to detect mycobacteria in different types of clinical samples. Out of 1,770 cultured clinical samples (1,519 of respiratory origin and 251 of nonrespiratory origin), mycobacteria were isolated in 156 samples (135 *M. tuberculosis* complex, 8 *M. chelonae*, 6 *M. kansasii*, 4 *M. fortuitum*, 2 *M. gordonae,* and 1 *M. marinum*) by at least one of the methods used. The BACTEC MGIT 960 System proved to be the most sensitive method (86.5%), especially in the detection of *M. tuberculosis* complex (89.1%). However, Löwenstein–Jensen culture was the most sensitive (76.2%) to detect nontuberculous mycobacteria. The BACTEC MGIT 960 System showed the lowest mean detection time for mycobacterial growth (15.3 days), significantly shorter than the other two methods. Highest sensitivity (95.5%) and specificity (99.6%) values were obtained using the BACTEC MGIT 960 System with the Löwenstein–Jensen culture method, which was also the only combination capable of detecting 100% of the nontuberculous mycobacteria.

Keywords: Mycobacteria, MB/BacT ALERT 3D, BACTEC MGIT 960, Löwenstein–Jensen

Mycobacteria are responsible for numerous infections with high morbi-mortality and represent a worldwide health problem. The appearance over recent years of strains resistant to antituberculosis antibiotics is of special concern [18].

Microbiological diagnosis of diseases caused by mycobacteria is essential and should be fast and effective to prevent contagions and optimize the management of infections [11]. The use of fluorochromes in direct sample staining, the simultaneous use of solid and liquid culture media, the monitoring of bacterial growth using automated incubation and reading methods, and the use of genetic probes for identification have all served to improve sensitivity and reduce diagnosis times [6].

Besides offering higher sensitivity than microscopic examination, sample culturing permits species identification, study of susceptibility to antituberculosis antibiotics, monitoring of treatment, and determination of patient recovery based on negative cultures. The utilization of a solid culture medium in conjunction with a liquid medium has been recommended [5, 17] to improve the isolation of mycobacteria from clinical samples.

Numerous automated incubation and reading methods are available to monitor bacterial growth in liquid media. Some are based on radiometric detection methods, such as the Radiometric BACTEC 460 System [1], and some on colorimetric methods that detect bacterial CO_2 production, such as MB/BacT ALERT 3D System [12]. Others use pressure sensors or fluorometric methods to detect bacterial O_2 consumption, such as the ESP Culture System II [14] and BACTEC MGIT 960 System [16], respectively.

A considerable number of these methods provide similar times to detection, with fully automated instruments or without the need for any instrumentation. In our laboratory, we have used the MB/BacT ALERT 3D System for some years for rapid culture of mycobacteria. In January 2008, we have installed the BACTEC MGIT 960 System; for this reason the objective of this study was to assess the capacity of the MB/BacT ALERT 3D System (bioMérieux, Marcy-l'Etoile, France), BACTEC MGIT 960 System (Becton Dickinson, Franklin Lakes, NJ, U.S.A.), and Löwenstein–Jensen culture medium to isolate mycobacteria in clinical samples of respiratory and extrapulmonary origin

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and to determine the time required to detect bacterial growth in each method.

Materials and Methods

Specimen Collection and Treatment

The study included 1,770 clinical samples obtained from 696 patients consecutively referred for mycobacterial culture at the Microbiology Department of our hospital between January and July 2008. Samples were obtained from sputum (1,388), bronchoaspiration (74), bronchoalveolar lavage (9), pleural fluids (48), nonpleural sterile pericardial, peritoneal, synovial, and cerebrospinal fluids (33 in total), urine (153), gastric fluids (41), biopsies from different tissues (12), and miscellaneous sources, including abscesses, exudates, and adenopathies (12). Blood samples were excluded from this study.

Specimens collected from contaminated sites were liquefied and decontaminated with an equal volume of *N*-acetyl-L-cysteine and 3% sodium hydroxide for 20 min at room temperature. The tubes were then centrifuged at 3,500 ×*g* for 20 min. The supernatant fluid was decanted carefully, and the sediment was retained. The sediment was suspended in 3 ml of sterile phosphate-buffered saline (0.067 M, pH 6.8). Specimens collected from sterile sites were concentrated by centrifugation without prior decontamination. The resulting suspension was used for smear preparation and inoculated into the three culture media.

Smear Preparation

Smears were prepared from all samples and examined for the presence of acid-fast bacilli (AFB). All smears were stained with auramine-rhodamine and studied under a fluorescent microscope. Samples were considered positive when they showed the presence of at least 2–3 AFB/10 fields with a 200× zoom lens.

Inoculation and Cultivation of Samples

Exactly 0.5 ml of the digested and decontaminated suspension was inoculated into BacT/ALERT MP (bioMérieux) and BBL MGIT (Becton Dickinson) culture tubes. In both cases, 0.5 ml of antimicrobial solution was added to the tubes before sample inoculation to reduce the incidence of contamination from other bacteria. In the case of BacT/ALERT MP, a lyophilized supplement (MB/BacT) was added, consisting of amphotericin B, azlocillin, nalidixic acid, polymyxin B, trimethoprim, and vancomycin reconstituted in purified water, oleic acid, glycerol, amaranth, and bovine seroalbumin in accordance with the manufacturer's recommendations. In BBL MGIT tubes, a lyophilized supplement (BBL MGIT PANTA) was added, consisting of amphotericin B, azlocillin, nalidixic acid, polymyxin B, and trimethoprim reconstituted in purified water, oleic acid, bovine seroalbumin, dextrose, polyoxyethylene stearate, and catalase in accordance with the manufacturer's recommendations.

In addition, 0.5 ml of all samples was inoculated into Löwenstein–Jensen solid medium and incubated at 37°C for 50 days, with examinations at least once a week.

After sample inoculation, BacT/ALERT MP tubes were introduced into the MB/BacT ALERT 3D instrument, and BBL MGIT tubes into the BACTEC MGIT 960 instrument, and incubated at 37°C for 50 days. Continuous monitoring was maintained in both methods, and cultures remained in the reading-incubator with no additional handling until either positivity or negativity was recorded after the predetermined incubation period. In both methods, a series of algorithms were used to determine presumptive positivity and to alert the operator to the presence and location of positive tubes.

Any sample identified as positive by the instrument (MB/BacT ALERT 3D System or BACTEC MGIT 960 System) was removed from it, and to be confirmed, a smear from liquid media was prepared and tested for the presence of AFB by Ziehl–Neelsen (ZN) staining. If staining confirmed the presence of AFB, the result was considered positive (true positive by the instrument). If staining did not reveal AFB, a subculture on Middlebrook 7H11 agar was performed to control growth and purity, and the tube was re-incubated at 37°C in a heater (not in the instrument) for a further 50 days, after which the ZN staining was repeated. Samples showing mycobacterial growth in Middlebrook 7H11 agar and/or presence of AFB by ZN staining after the 50-day incubation were considered positive (true positive by the instrument). Any sample initially identified as positive by the instrument but showing no presence of AFB by staining or mycobacterial growth in Middlebrook 7H11 agar, was considered negative (false positive by the instrument).

Samples were also considered positive if colonies were observed in Löwenstein–Jensen medium and AFB presence of these colonies was confirmed by ZN staining (true positive by solid media).

Samples were considered negative if mycobacterial growth was not detected by any automated method or no growth was observed in Löwenstein–Jensen medium after 50 days (true negative in all methods).

Mycobacterial Species Identification

All samples that were positive in any of the three methods were identified by specific nucleic acid probes (AccuProbe; Gen Probe, San Diego, CA, U.S.A.) following the manufacturer's recommendations after DNA extraction by sonication. This method identifies *Mycobacterium tuberculosis* complex, *Mycobacterium avium-intracellulare*, *Mycobacterium kansasii,* and *Mycobacterium gordonae* from solid or liquid cultures by means of hybridization with mycobacterial ribosomal RNA [3]. When the mycobacteria could not be identified by using specific probes, identification was done by means of biochemical tests (niacin test, nitrate reductase, catalase test, Tween 80 hydrolysis, pyrazinamidase and urease tests, among others) [8].

Results Interpretation and Statistical Analysis

A sample was considered true negative (TN) when no mycobacterial growth took place in any of the three culture methods after 50 days of incubation.

A sample was considered true positive (TP) when the presence of AFB was confirmed in at least one of the culture methods and the species was subsequently identified.

It was felt that a sample was false negative (FN) for some methods when the same sample was positive by any of the others. If a presumptively positive culture medium was positive for a bacterium that was not AFB by either ZN staining or subculture in Middlebrook 7H11 agar after the incubation period, this was recorded as a false positive (FP) for the method owing to contamination from other non-acid-fast bacteria. The confirmation of contamination for a bacterium that was not AFB was conducted by subculturing on blood-agar. Hence, the FP rate for each automated method was calculated as the number of tubes that were instrument-positive but after smear and

subculture of the liquid media were found to be ZN-negative and subculture-negative for mycobacteria.

The finding of bacterial growth on Löwenstein–Jensen medium with no presence of AFB by ZN staining was considered to be due to bacterial contamination and a negative result for the presence of mycobacteria. As reported above, this was considered a TN when no growth was observed in either of the other methods and an FN if mycobacteria were isolated in either of them.

A combination of results of solid and liquid media was regarded as the "gold standard" to measure sensitivity, specificity, and predictive values.

The statistical difference in the number of days required to recover mycobacteria by each method was determined by Student's *t* test. A. *P* value ≤0.05 was considered significant.

RESULTS

A total of 1,770 clinical samples was received during the study period. Mycobacteria were isolated by at least one of the three methods used in 156 samples (8.8%) obtained from 79 patients (11.4%). Out of these 156 samples, 141 (90.4%) were of respiratory origin, including 131 sputum (112 *M. tuberculosis*, 8 *M. chelonae*, 6 *M. kansasii*, 4 *M. fortuitum*, and 1 *M. gordonae*), 7 bronchoaspiration (all *M. tuberculosis*), 2 bronchoalveolar lavage (both *M. tuberculosis*), and 1 pleural fluid *(M. tuberculosis*) samples. The remaining 15 samples were of extrapulmonary origin: 8 urinary (all *M. tuberculosis*), 2 gastric fluid (*M. tuberculosis* and *M. gordonae*), 3 biopsy (2 *M. tuberculosis* and 1 *M. marinum*), 1 adenopathy (*M. tuberculosis*), and 1 synovial fluid (*M. tuberculosis*) samples.

None of the three methods cultured all of the clinical isolates, but each method isolated mycobacteria that were not detected by either of the others.

Table 1 shows the different types of samples according to origin (first column); number (and percentage) of samples in which culture was positive (in any of the three methods) or negative (in all three methods at once), and the identification of mycobacteria in the first case (third column); the number of samples (and percentage) that were positive or negative smear (fifth column); and finally, the number of samples (and percentage in relation to the result of the smear) in

Table 1. Isolation rate of mycobacteria in relation to acid-fast bacilli smear and type of sample.

Type of sample (No.)	Mycobacteria	No. (%)	Smear result	No. (%) of samples	No. (%) of positive cultures		
					Löwenstein–Jensen	MB/BacT ALERT 3D	BACTEC MGIT 960
All samples (1,770)	Mycobacteria not isolated	1,614 (91.2)	Positive	7 (0.4)	-	-	-
			Negative	1,607 (99.6)	-	-	-
	M. tuberculosis complex	135 (7.6)	Positive	56 (41.5)	45 (80.4)	53 (94.6)	52 (92.9)
			Negative	79 (58.5)	63 (79.7)	64 (81.0)	68 (86.1)
	Nontuberculous mycobacteria	21 (1.2)	Positive	6 (28.6)	6 (100)	5 (83.3)	6 (100)
			Negative	15 (71.4)	10 (66.7)	2 (13.3)	9 (60.0)
Samples of respiratory origin[a] (1519)	Mycobacteria not isolated	1,378 (90.7)	Positive	7 (0.5)	-	-	-
			Negative	1,371 (99.5)	-	-	-
	M. tuberculosis complex	122 (8.0)	Positive	54 (44.3)	43 (79.6)	51 (94.4)	50 (92.6)
			Negative	68 (55.7)	53 (77.9)	54 (79.4)	58 (85.3)
	Nontuberculous mycobacteria	19 (1.3)	Positive	6 (31.6)	6 (100)	5 (83.3)	6 (100)
			Negative	13 (68.4)	8 (61.5)	2 (15.4)	9 (69.2)
Sterile fluids[b] (33)	Mycobacteria not isolated	32 (97.0)	Positive	0	-	-	-
			Negative	32 (100)	-	-	-
	M. tuberculosis complex	1 (3.0)	Positive	0	-	-	-
			Negative	1 (100)	1 (100)	1 (100)	1 (100)
	Nontuberculous mycobacteria	0	Positive	-	-	-	-
			Negative	-	-	-	-
Other samples of extrapulmonary origin[c] (218)	Mycobacteria not isolated	204 (93.6)	Positive	0	-	-	-
			Negative	204 (100)	-	-	-
	M. tuberculosis complex	12 (5.5)	Positive	2 (16.7)	2 (100)	2 (100)	2 (100)
			Negative	10 (83.3)	9 (90.0)	9 (90.0)	9 (90.0)
	Nontuberculous mycobacteria	2 (0.9)	Positive	0	-	-	-
			Negative	2 (100)	2 (100)	0	0

[a]Included specimens from sputa, bronchoaspirations, bronchoalveolar lavages, and pleural fluids.
[b]Included specimens from pericardial, peritoneal, synovial, and cerebrospinal fluids.
[c]Included specimens from urine, gastric fluids, biopsies, abscesses, adenopathies, and exudates.

Table 2. Isolation of mycobacteria with Löwenstein–Jensen medium, the MB/BacT ALERT 3D System, and the BACTEC MGIT 960 System.

Mycobacteria	Total no. (%) of isolates recovered in all media	No. (%) of isolates recovered in				
		Löwenstein–Jensen	MB/BacT ALERT 3D	BACTEC MGIT 960	MB/BacT ALERT 3D plus LJ	BACTEC MGIT 960 plus LJ
All mycobacteria	156 (100)	124 (79.5)	124 (79.5)	135 (86.5)	143 (91.7)	149 (95.5)
M. tuberculosis complex	135 (86.6)	108 (80)	117 (86.7)	120 (89.1)	126 (93.3)	128 (94.8)
M. chelonae	8 (5.1)	5 (62.5)	1 (12.5)	7 (87.5)	5 (62.5)	8 (100)
M. kansasii	6 (3.8)	6 (100)	5 (83.3)	5 (83.3)	6 (100)	6 (100)
M. fortuitum	4 (2.6)	2 (50)	1 (25)	3 (75)	3 (75)	4 (100)
M. gordonae	2 (1.3)	2 (100)	0	0	2 (100)	2 (100)
M. marinum	1 (0.6)	1 (100)	0	0	1 (100)	1 (100)

LJ: Löwenstein–Jensen medium.

which the cultures were positive (for each of the three methods studied).

This table shows that 0.4% of negative samples were positive under bacilloscopy (bacilloscopy specificity of 99.6%), whereas 60.3% of samples that were initially negative under bacilloscopy proved to be positive (bacilloscopy sensitivity of 39.7%). These false negatives were more frequent in cases of nontuberculous mycobacteria (71.4%) versus *M. tuberculosis* complex (58.5%) and in samples of extrapulmonary versus respiratory origin.

All three methods yielded a very high percentage of positive isolates in samples with negative bacilloscopy; the BACTEC MGIT 960 System showed a higher percentage in cases of *M. tuberculosis* complex (86.1%) and in samples of respiratory origin (82.7%), whereas culture in Löwenstein–Jensen medium showed a higher percentage in cases of nontuberculous mycobacteria (66.7%).

Table 2 gives the number of isolates (and percentage) of each species obtained by the methods under study, both separately and considering liquid- and solid-medium culture methods together. The BACTEC MGIT 960 System detected the highest number of mycobacterial isolates (86.5%), mainly *M. tuberculosis* complex (89.1% of this species). The largest percentage of nontuberculous mycobacteria isolates was obtained by culture in Löwenstein–Jensen medium (76.2%). A higher percentage of isolates was detected by combining the BACTEC MGIT 960 and Löwenstein–Jensen methods (95.5%) than by combining the MB/BacT ALERT 3D and Löwenstein–Jensen methods (91.7%), both for *M. tuberculosis* complex (94.8% vs. 93.3%, respectively) and nontuberculous (100% vs. 89.0%) species.

The rate of contamination by non acid-fast bacteria was 7.1% (126/1770) for culture in Löwenstein–Jensen medium (all considered TNs after ZN staining demonstrated absence of AFB), 2.3% (40/1770) for the MB/BacT ALERT 3D System, and 0.3% (6/1770) for the BACTEC MGIT 960 System (all 46 considered FPs).

Table 3 shows sensitivity, specificity, and positive and negative predictive values of each method under study, both separately and considering liquid- and solid-medium culture methods together. The highest sensitivity and negative predictive values to detect mycobacteria were shown by the BACTEC MGIT 960 System, both when used alone and with Löwenstein–Jensen medium. This combination was capable of detecting 100% of nontuberculous mycobacteria. The highest specificity and positive predictive values were shown by the Löwenstein–Jensen method, which produced no false positives.

Finally, Table 4 shows the growth detection time (mean days, standard deviation, and range) in the Löwenstein–Jensen medium, the MB/BacT ALERT 3D System, and the BACTEC MGIT 960 System. Growth detection times for mycobacteria were significantly shorter in liquid-medium than in solid-medium culture ($p<0.05$ in both cases). Detection time was shortest with the BACTEC MGIT

Table 3. Sensitivity, specificity, and positive and negative predictive values (%) of each method under study.

Culture in	Sensitivity	Specificity	PPV	NPV
Löwenstein–Jensen	79.5	100.0	100.0	98.1
MB/BacT ALERT 3D	79.5	97.5	75.6	98.0
BACTEC MGIT 960	86.5	99.6	95.7	98.7
MB/BacT ALERT 3D plus LJ	91.7	97.5	78.1	99.2
BACTEC MGIT 960 plus LJ	95.5	99.6	96.1	99.6

PPV, Positive predictive value; NPV, negative predictive value.

Table 4. Detection time in the Löwenstein–Jensen medium, the MB/BacT ALERT 3D System, and the BACTEC MGIT 960 System.

Mycobacteria	Detection time: mean days±standard deviation (range) in		
	Löwenstein–Jensen	MB/BacT ALERT 3D	BACTEC MGIT 960
All mycobacteria	32.4±11.9 (10–50)	20.2±9.0 (8–48)	15.1±6.4 (5–41)
M. tuberculosis complex	32.6±11.8 (10–50)	20.1±8.6 (8–48)	15.3±6.1 (5–39)
M. chelonae	21.6±5.3 (13–25)	12[a]	12.6±0.5 (12–13)
M. kansasii	29.8±10.8 (17–39)	19.2±13.0 (9–39)	12±5.6 (8–21)
M. fortuitum	49.5±0.7 (49–50)[b]	44[a]	20.3±18.9 (5–41)
M. gordonae	43±9.9 (36–50)[b]	ND	ND
M. marinum	19[a]	ND	ND

[a]Only one isolate with this identification was found in this method.
[b]Only two isolates with this identification were found in this method.
ND: No samples with this identification were found in this method.

960 System, both for *M. tuberculosis* complex (mean of 15.3 days) and nontuberculous mycobacteria (mean of 15.1 days), and was significantly shorter than with MB/BacT ALERT 3D System, the other liquid-medium culture method.

DISCUSSION

This study compared the capacity of Löwenstein–Jensen medium, MB/BacT ALERT 3D System, and BACTEC MGIT 960 System to recover mycobacteria from different types of clinical samples. The first two methods recovered 79.5% of mycobacteria, whereas the BACTEC MGIT 960 System recovered 86.5%. Therefore, the performance of the BACTEC MGIT 960 System proved superior to that of the other two methods tested.

Numerous studies have favorably evaluated the MB/BacT ALERT 3D and BACTEC MGIT 960 liquid-medium culture methods [4, 13], but culture in solid medium still plays an important role in the isolation of mycobacteria from clinical samples and is recommended for use alongside a liquid medium by the Center for Disease Control [5]. In the present study, the detection rate was increased when the liquid-medium methods were combined with the Löwenstein–Jensen solid-medium method, and the highest overall recovery rate (95.5%) was obtained by the combination of the BACTEC MGIT 960 System with Löwenstein–Jensen medium. Although none of the methods could isolate all of the mycobacteria, this combination achieved 100% recovery of nontuberculous mycobacterial isolates in the present series. The positive predictive value of the combination BACTEC MGIT 960 plus Löwenstein–Jensen was considerably higher than the MB/BacT ALERT 3D plus Löwenstein–Jensen. However, the negative predictive values of these combinations were similar.

Culture in Löwenstein–Jensen medium has been widely found to offer low sensitivity for the recovery of mycobacteria species, as in the present study. Thus, Rishi *et al*. [13] obtained a sensitivity value of 63.95% with a contamination rate of 27.2%, although Brunello *et al*. [4] described an especially high sensitivity value of 95.9% and a low contamination rate of 5%.

Reported sensitivity values for the MB/BacT ALERT 3D System range from 78% to 99%, with contamination rates of 4% to 7% [2, 15]. As in this study, this method has consistently demonstrated a higher capacity to detect *M. tuberculosis* complex (91.3%) than atypical mycobacteria (38.0%) [12]. The detection capacity was considerably increased when this method was used in combination with Löwenstein–Jensen medium, both for *M. tuberculosis* complex (increase from 88.5% to 91.7%), and more markedly, for nontuberculous species (increase from 62.5% to 75%) [2].

The sensitivity of the BACTEC MGIT 960 System has been reported variously to be 98.1% by Rishi *et al*. [13], with a contamination rate of 13.4%; 82.5% by Alcaide *et al*. [2] with a 3.3% contamination rate; and 80% by Hanna *et al*. [7] with an 8.1% contamination rate. Higher sensitivity values for both *M. tuberculosis* complex and nontuberculous mycobacteria were reported when this method was used in combination with culture in Löwenstein–Jensen medium [2, 7].

The time taken to isolate mycobacteria is another important consideration and was shortest with the BACTEC MGIT 960 System in this study. Less time was required to detect bacterial growth in all methods when the sample evidenced the presence of AFB, as previously reported [9, 10].

A major drawback of the BACTEC MGIT 960 System appears to be the handling required to add the antibiotic and enrichment supplements. Pipettes are used for this purpose rather than needles or syringes, involving the unscrewing of the tube caps, which does not occur in the MB/BacT ALERT 3D System. Nevertheless, despite this increased handling, a low contamination rate was obtained with this method in the present study.

In conclusion, the BACTEC MGIT 960 System is a suitable method for recovering tuberculous and nontuberculous mycobacteria from clinical samples of respiratory or nonrespiratory origins, recovering them quickly with a

very low rate of contamination from nonmycobacterial microorganisms. All of these benefits are enhanced when this method is used in combination with a solid-medium culture method such as Löwenstein–Jensen medium.

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