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Detection of *Mycobacterium tuberculosis* from respiratory samples with the liquid culture system MB/BacT and verified by PCR

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ABSTRACT

Objective. To assess the performance in the clinical setting of the MB/BacT system for isolation of *Mycobacterium tuberculosis* and to verify by PCR. **Material and methods.** The study included 272 sputum samples from 208 patients with the presumptive diagnosis of pulmonary tuberculosis. ZN was made, culture in Löwenstein-Jensen medium, MB/BacT and PCR. **Results.** Thirty-nine samples were positive by culture in Löwenstein-Jensen, and 42 using the MB/BacT system. Positive cultures in the MB/BacT system were verified by acid-fast bacilli staining and PCR. Mycobacterial identification in the MB/BacT took 8 to 46 days (mean 16 days), while the Löwenstein-Jensen culture ranged between 21 and 63 days (mean 35 days). These results show that the MB/BacT semiautomated system is reliable and faster than the manual culture method and can be used as an alternative for the primary identification of *Mycobacterium tuberculosis*. The PCR assay allows the fast and exact identification of *Mycobacterium tuberculosis* directly from positive liquid medium.

Key words. *Mycobacterium tuberculosis*. Liquid culture system MB/BacT. PCR.

Detección de* Mycobacterium tuberculosis *de muestras respiratorias con el sistema de cultivo líquido MB/BacT y verificado por PCR

RESUMEN

Objetivo. *Evaluar en el marco clínico el sistema MB/BacT para el aislamiento de* Mycobacterium tuberculosis *y su verificación mediante PCR.* ***Material y métodos.*** *El estudio incluyó 272 muestras de esputo provenientes de 208 pacientes con el diagnóstico presuntivo de tuberculosis pulmonar. Se realizó la tinción de ZN, así como cultivo en el medio de Löwenstein-Jensen, MB/BacT y PCR.* ***Resultados.*** *Por cultivo en Löwenstein-Jensen resultaron positivas 39 muestras y 42 utilizando el sistema MB/BacT. Los cultivos positivos en el sistema MB/BacT se verificaron con tinción para bacilos ácido alcohol resistentes y por PCR. El desarrollo micobacteriano en MB/BacT se presentó de ocho a 46 días (media 16 días), mientras que en el medio Löwenstein-Jensen el rango fue de 21 a 63 días (media 35 días). Estos resultados demuestran que el sistema semiautomatizado MB/BacT es confiable y más rápido que el método de cultivo manual y puede utilizarse como una buena alternativa para el cultivo primario de* Mycobacterium tuberculosis. *El ensayo de PCR también permite la identificación exacta y rápida de* Mycobacterium tuberculosis *directamente del medio líquido positivo.*

Palabras clave. Mycobacterium tuberculosis. *Sistema de cultivo líquido MB/BacT. PCR.*

INTRODUCTION

Tuberculosis is an endemic problem in many poor countries of the world, and considered reemergent in certain developed countries where the morbidity rates have increased annually around 20%.[1] This resurgence is mainly due to migration, co-infection with the human immunodeficiency virus (HIV), and the dissemination of multidrug resistant strains.[2] According to the World Health Organization (WHO), approximately 10 million cases of tuberculosis are detected annually worldwide, becoming the most common cause of death due a single infectious agent, responsible for 3-4 million deaths annually.[3,4]

Pulmonary tuberculosis is the most frequent clinical form of tuberculosis and it is caused by *Mycobacterium tuberculosis*. Nevertheless, atypical mycobacterial infections are rising due to the coinfection with HIV. The disease is transmitted through contact with subjects with active pulmonary tuberculosis and persistent coughing. The bacilli reach the alveoli where the cell immune response generally limits the dissemination of the bacilli by the formation of granulomas.[2] However, the infectious process may reactivate when the immune system is debilitated, when the patient suffer other diseases such as diabetes, alcoholism, renal failure or lymphomas.

The epidemiological control of tuberculosis depends on the opportune identification of acid-fast bacilli in patients with persistent productive cough and their adequate treatment to avoid bacterial spread between their contacts.[2] However, on occasions the diagnosis of tuberculosis may be difficult since some patients may have vague clinical symptoms and *M. tuberculosis* may not always be possible to isolate.[5-7]

For many years, the diagnosis of pulmonary tuberculosis has been based on the identification of acid-fast bacilli in sputum samples from patients with productive cough. However, acid-fast staining is not specific and between 40-60% false negative results have been reported in these patients.[2] Therefore, several methods have been reported with different specificity and sensitivity for the detection of *M. tuberculosis* nucleic acids from liquid cultures,[8,9] as well as for the direct bacterial detection in clinical samples.[10-17] Other diagnostic methods based on the detection of circulating antibodies have been attempted, but until now they reveal low specificity, and are recommended as screening tests.

Therefore, the culture of *M. tuberculosis* continues considered as the bacteriologic "gold standard" for the diagnosis of tuberculosis and is required for drug sensitivity testing. Generally, the culture should be performed in a combination of solid and liquid media for the primary isolation of the bacteria with observation time not exceeding 21 to 30 days after the recollection of the sample.[18,19] In 1977, a semi-automated method was developed for the detection and growth of *M. tuberculosis* in a liquid medium (7H12) culture in bottles containing ^{14}C-marked palmitic acid. This method detected the release of radioactive CO_2, the product of the bacterial metabolic activity in an ion chamber system.[20] It was demonstrated that this system detects the presence of 200 viable microorganisms in an average of 7 to 14 days. The automated equipment named BACTEC TB460 has been used in the clinical setting with excellent results. However, it requires the use of radioactive material making it inaccessible to most clinical testing laboratories in high-prevalence countries.[20-29]

Other semi-automatic culture system with similar principle is based on the measurement of the CO_2, released into the medium by actively growing mycobacteria, through a gas-permeable sensor containing a colorimetric indicator embedded at the bottom of the culture vials. This system known as MB/BacT™ does not require radioactive material, and color changes are monitored though a reflectometric detection unit contained within each incubating spot of the instrument. This system has been reported to detect between 200 to 300 live microorganisms in 1-2 weeks.[21-26,28,30,31]

The current study compared the diagnoses achieved by culture in Löwenstein-Jensen and the automated MB/BacT method™ in a hospital setting. In addition, PCR and acid-fast staining was done directly on the MB/BacT liquid medium immediately after the culture bottles were marked as positive by the instrument to prove the specificity of the method.

MATERIAL AND METHODS

Clinical sample processing

A total of 272 sputum samples were obtained from 208 patients, and were analyzed 110 (62%) males and 98 (38%) females from the General Hospital at the "La Raza" National Medical Center, IMSS, Mexico City. One sputum sample was received from 165 out of the 208 patients (79.2%), two samples from 32 patients (15.4%) and three or more samples from seven patients (5.4%), 214 (78.7%) sample came from patients with the presumptive diagnosis of pulmona-

ry tuberculosis and the remaining 58 (21.3%), with diagnosis of pneumonia and pleural exudation.

The samples were digested and decontaminated using Petroff's method,[32] and then were divided for Ziehl-Neelsen staining (ZN), and culture in Löwenstein-Jensen medium and in MB/BacT.

This system uses of bottles containing 10 mL of Middlebrook 7H9 broth enriched with casein, bovine serum albumin, and catalase. After decontamination and concentration of the samples, the sediment was neutralized and a 0.5 mL volume of the sediment inoculated in the liquid and 0.1 mL in the solid media. The MB/BacT bottles were supplemented with 0.5 mL antibiotic supplement (Antibiotic Supplement Kit MB/BacT Cat No. 259760) (Amphotericin B, azlocilin, nalidixic acid, polymyxin B, trimethoprim and vancomycin) (Biomeriux), reconstituted with 10 mL of the MB reconstituting fluid (Tween 80, glycerol, amaranth and purified water) following the manufacturers instructions (BioMerieux). After the inoculation, the bottles were introduced in the MB/BacT instrument (BioMerieux) and incubated at 35 ºC for 8 weeks. The MB/BacT bottles were analyzed every 10 minutes using a computerized software system and the solid medium cultures were examined weekly. The cultures positive for microbial growth were analyzed using Ziehl-Neelsen (ZN) staining and in cases positive for acid-fast bacilli, conventional identification methods were then followed.[33] PCR amplification was also carried out as described below.

Polymerase Chain Reaction (PCR)

The mycobacterial strain used as a positive control was *M. tuberculosis* H37Rv, and a tube without DNA for negative control. The DNA was isolated with guanidine isotiocianate and phenol, using 500 µL of the TRIzol reagent (Gibco BRL).[34] The DNA was resuspended in 50 µL of distilled water after precipitating with 75% ethanol. This solution was heated at 55 ºC for 20 minutes. Using 260/280 nm absorbance ratio as measure of purity. A multiplex PCR was performed in order to amplify three different mycobacterial genes simultaneously. Five µL were used for the multiplex PCR amplification of the genes encoding for the 32 kDa protein,[35] which are specific for the genus *Mycobacterium*, the protein MTP40,[36,37] specific for specie *tuberculosis* and the IS6110 insertion sequence,[38] which identify *M. tuberculosis* Complex. This multiplex PCR method is a rapid, sensitive, and specific tool for the identification and differentiation of various mycobacterial strains in a single-step assay. The primer sequences used for the *mtp40* gene amplification were: PT1 (5'CGG CAA CGC GCC GTC GGT GG) and PT2 (5'CCC CCC ACG GCA CCG CCG GG) which results in a 396 bp fragment.[36] The amplification of the IS6110 insertion element was performed with the primers IS5 (5'CGG AGA CGG TGC GTA AGT GG) and IS6 (5'GAT GGA CCG CCA GGG CTT GC), which amplify a 984 bp fragment. The primers for the 32 kDa alpha antigen encoding gene present in all mycobacteria were: MT1 (5´TTC CTG ACC AGC GAG CTG CCG) and MT2 bp (39,40-42). All of the reactions were performed in a final volume of 50 µL containing 100 ng of purified DNA, 1x reaction buffer, 2.5 U of Taq polymerase, 0.2 mM dNTPs and 20 pM of each primer. A thermocycler (Biometra) was used with an initial denaturalization at 94 ºC for 5 minutes, followed by 35 cycles of denaturalization at 94 ºC for a minute, alignment at 71 ºC for 2 minutes, and an extension at 72 ºC for 3 minutes and a final extension at 72 ºC for 10 minutes.

To increase the sensitivity of the amplification, a nested PCR was performed amplifying an internal segment of the *mtp40* gene.[36] The internal primers for the second amplification corresponded to the 44 to 65 (PT3, 5'-CAC CAC GTT AGG GAT GCA CTG C-3') and 244 to 265 nucleotides (PT4, 5'-CTG ATG GTC TCC GAC ACG TTC G-3') with an expected product of 223 bp.[43] Five µL of the multiplex PCR product were transferred to 45 µL of a premixed solution containing the PCR reagents at the same concentration described.[37] The amplification was repeated for 30 cycles using the same time and temperature parameters as described above, except for an alignment at 75 ºC for 2 minutes, an extension at 72 ºC for 2 minutes and a final extension at 72 ºC for 7 minutes. After the amplification, 5 µL of the PCR products were analyzed electrophoretically in 1.5% agarose gells containing 0.5 µg/mL ethidium bromide.

The main aim of the study was to compare the MB/BacT method *vs.* the traditional culture (L-J – gold standard). The PCR was used to corroborate the positive results. Both PCR tests were used in order to detect all mycobacterias.

Statistical analysis

To determine the level of significance during recovery, the Wilcoxon rank sum test was used for dependent samples.[44] Using the data generated from the assessment, differences between diagnostic methods were determined using Student's t test for paired samples. Contigency tables were used to

compare the diagnostic test against the gold standard, with which sensitivity, specificity and positive and negative predictive values were obtained.

RESULTS

Acid-fast bacilli were demonstrated in 38 of 272 samples analyzed (13.9%), 39 (14.3%) grew mycobacteria in Löwenstein-Jensen medium (Table 1) and 42 (15.4%) were positive by the MB/BacT system. In almost all positive cultures tested in the MB/BacT system, the formation of a acid fast bacilli cords from the culture supernatant was confirmed when staining with ZN, only one of them did contain noncording AFB, corresponding to *Mycobacterium bovis*. The recovery rate was 97.6% for MB/BacT and 92.8% for Löwenstein-Jensen, with 38 positive AFB samples, corresponding all of them to *Mycobacterium tuberculosis*. When comparing the acid-fast bacilli test and MB/BacT, with Löwenstein-Jensen culture considered the traditional bacteriologic gold standard, for 272 patients, it was found that the acid-fast test had a lower sensitivity (71.4%) and MB/BacT (100% sensitivity) (Table 2).

Table 1. Detection results of *Mycobacterium tuberculosis* according to the method used.

Diagnostic method	Results		Total number of samples analyzed
	Positive	Negative	
Acid-fast staining	38	234	272
MB/BacT	42	230	272
Löwenstein-Jensen Culture	39	233	272

$p < 0.0001$ between the days of culture. Using t of Student for matched samples.

Table 2. Behavior of the detection methods acid-fast staining and MB/BacT system for *Mycobacterium tuberculosis* in comparison with the Löwenstein-Jensen culture method.

Diagnostic method	Löwenstein-Jensen AFB	Löwenstein-Jensen MB/BacT	Culture* PCR
Sensitivity	71.4	97.4	96
Specificity	95	98	100
PPV	71	90	100
NPV	95	98	100
Eficacy	92	98	96

* Culture: Löwenstein-Jensen and MB/BacT.

It is important to note that the presence of *Mycobacterium tuberculosis* was confirmed through PCR from culture medium in the MB/BacT vials; by a multiplex followed a nested PCR. The multiplex PCR, is able to detect a mycobacteria belonging to the *M. tuberculosis* complex, with a sensitivity threshold of 2×10^6 bacilli/mL, 2×10^5 bacilli/mL for the genus *Mycobacterium*, and 2×10^4 bacilli/mL for the species *tuberculosis*, whereas the nested PCR increases the sensitivity in 1×10^4. Therefore, the use of both amplifications offers an advantage when compared with the acid fast ZN staining, since this method is only able to detect AFB. Using the PCR it is possible to define *M. tuberculosis complex, M. tuberculosis and* atypic mycobacteria. An initially Löwenstein-Jensen TB-negative patient was found to be positive by MB/BacT. Only a sample from a previously treated patient was not MB/BacT positive. The specific weight that each method may

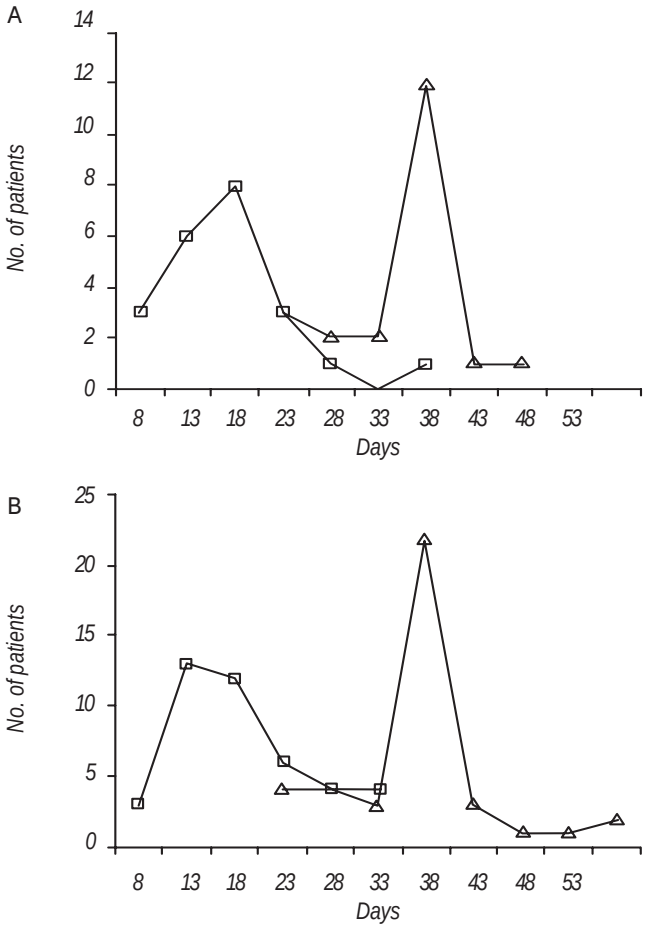

Figure 1. Recovery rates of Mycobacterium tuberculosis *according to the method used. A. According to the samples analyzed B. According to the patients studied. (■) MB/BacT System (▲) Löwenstein-Jensen media culture.*

have in the diagnosis of tuberculosis was not determined, because each method may be used independently in a clinical situation.

Of the 272 cultures tested using the MB/BacT system, 42 were positive between the 8th and 46th day after seeding, with a mean of 18 days and a median of 16. In contrast, with the Löwenstein-Jensen medium, 39 cultures were positive with evident growths between 21 and 63 days, with a mean and median of 35 days (Figure 1A). Therefore, it took 17 days less to establish a diagnosis using the MB/BacT system ($p < 0.0005$). These data are very similar when considering the time to diagnosis per patient and by sample (Figure 1B). The occurrence of a false alarm due to sample contamination was 1.1% for MB/BacT, and 5% for Löwenstein-Jensen, they were evaluated by ZN. The most frequent contaminants were coccus in both methods.

DISCUSSION

Identification of patients with tuberculosis is key to the epidemiological control of the disease. Although many patients present classical clinical features, which include persistent cough with bloody sputum, and radiological alterations, such as consolidation areas, fibrosis, calcifications, cavities or pleural effusions, the diagnosis continues to represent an important medical problem because an important number of patients may have undefined symptoms. In practice, the detection of acid-fast bacilli in sputum from patients with persistent cough constitutes the most useful diagnostic test since it is a fast, simple, and inexpensive. Nonetheless, this method has low sensitivity and specificity, and serial assays are recommended to detect the majority of patients. On the other hand, serological tests still need to improve their reliability and those based on the amplification of mycobacterial nucleic acids are not generally used, because it is labor-intensive and difficult to implement for routine use in many clinical laboratories. In spite of the considerable development of commercially available assays and their advantage in shortening the development time necessary for diagnosis, it is not expected that nucleic acid amplification techniques substitute the culture for the definitive diagnosis of clinically important mycobacterial infections.[36] Consequently, culture continues to be the bacteriologic gold standard for the identification of *Mycobacterium tuberculosis*.

The isolation of *Mycobacterium tuberculosis* requires experienced personnel and installations with adequate biosafety measures. Generally, culture is performed using the Löwenstein-Jensen solid medium. However, in order to obtain an optimal bacterial growth from clinical samples a combination of liquid and solid medium is recommended. Several semi-automated methods for the detection and growth of *M. tuberculosis* in a liquid medium have been developed. The BACTEC™ equipment detects CO_2 produced by the bacterial metabolic activity. Nevertheless, it requires the use of radioactive material making it inaccessible to most clinical laboratories. On the other hand, the MB/BacT™ system has the advantage of not containing radiactivally-marked elements.[15]

This investigation included only respiratory samples. Although it was not the purpose of this work to determine the detection threshold for each method, most of the samples included could correspond to paucibacillary patients, since cultures and ZN staining were negative. However, the advantage of the multiplex-nested PCR has to be further evaluated in this situation and also in extrapulmonary tuberculosis.

Herein we compared the isolation efficiency and the bacterial growth time using the Löwenstein-Jensen medium with the MB/BacT system. Using the MB/BacT system, the bacterial growth was detected between 8-25 days of culture in 85% of the samples, and in the remaining 15% within 27 and 46 days, these may be multidrug resistant strains from paucibacillary patients which represent approximately half the time needed to detect bacterial growth with the Löwenstein-Jensen media. Moreover, the MB/BacT system detected three patients more than traditional culture, and the patient who presented the slowest bacterial development was restarting treatment after being considered cured.

The MB/BacT has the advantage of being a closed system, thus preventing laboratory technicians from bacterial exposure in order to observe the microscopic bacterial features, ZN staining was performed in samples obtained from the culture bottles. The characteristic cord like grouping of *M. tuberculosis* was noted in almost all cases. Although, unnecessary in normal clinical settings, this procedure could be useful when infections by atypical mycobacteria are suspected. Additionally, we used PCR amplification to detect mycobacterial DNA directly from the MB/BacT culture bottles immediately after the instrument detected them as positive. Only one patient resulted negative by this procedure, thus suggesting that it could be useful when the identification time has to be to reduced more.

It has been reported, that culture with the MB/BacT is able to increase the rate of positive results (10%) and decrease the mean culture time (9 to 14.7 days) when compared with the conventional culture

with Löwenstein-Jensen media.[45,46] Moreover, it has been demonstrated that the culture in the selective liquid Middlebrook media using the MB/BacT, is able to reach 100% sensitivity and specificity when compared with other traditional culture methods.[47,48] In other countries, positive samples have been identified after 13.7 to 17.5 days of culture, whereas it takes 24.2 days in average to obtain positive samples using egg-based media.[19,49]

Te mean detection time of *M. tuberculosis in* acid-fast staining positive samples, acid-fast staining negative samples and non-tuberculous mycobacteria was 11.5, 19.9 and 19.6 days, respectively,[11] and the proportion of positive samples was 35.3% using the MB/BacT and 31.6% with the Löwenstein-Jensen culture method.[50]

Taken together, these results suggest that the MB/BacT identification method has advantages over the traditional culture with the Löwenstein-Jensen media:

1. Faster identification of the bacteria.
2. Higher sensitivity and specificity.
3. The possibility to identify atypical mycobacterial, in smears which depend on the cord factor.
4. The possibility to perform PCR directly from the culture flasks to confirm atypical mycobacterias.
5. No additional sample preparation procedures are necessary.

Although number of samples included in this study was low, the evaluation is acceptable with a CI of 90%, based in a 1.10% prevalence of tuberculosis in the General Hospital at the "La Raza" National Medical Center, IMSS, Mexico City. We conclude that the MB/BacT system can be considered as a valuable alternative to the radiometric system, especially in those laboratories with restrictions concerning the use and disposal of radioactive wastes. In the PCR samples analyzed, in relation to the positive cultures both in Löwenstein-Jensen and MB/BacT, 96% sensitivity was found in agreement with that reported in the literature.[43] There was a false negative. Therefore, the PCR assay allows the fast and exact identification of *Mycobacterium tuberculosis* directly from positive liquid medium correlating with the formation of the FAB cord and therefore, represents an important technological advance in clinical mycobacteriology.

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