A Study Evaluating Conventional and Automated System of Culture for Isolation of Mycobacteria from Smear Negative Tuberculosis Samples with Special Reference to MPT 64 Antigen

Rehena Sarkar[1], Debalina Das[2], Soumick Sarkar[3]

[1]Department of Microbiology, Deben Mahata Government Medical College and Hospital, Purulia, West Bengal, India. [2]Department of Microbiology, College of Medicine and Sagore Dutta Hospital, Kamarhati, Kolkata, West Bengal, India. [3]Blood Bank, Jagannath Gupta Institute of Medical Sciences and Hospital, Budge, Kolkata, West Bengal, India.

ABSTRACT

BACKGROUND

India is among one of the major tuberculosis (TB) endemic countries of the world. The Centers for Disease Control and Prevention (CDC) guidelines mandate early species identification of *M. tuberculosis* complex as an effective countermeasure. The conventional culture method, which is the 'gold-standard' technique of mycobacterial isolation is time consuming, while the newer, automated, liquid medium-based culture systems like BACT / ALERT 3D have considerably shorter detection time and greater sensitivity. Other than isolation it is also important to differentially identify *Mycobacterium tuberculosis* complex (MTBC) from nontuberculous mycobacteria (NTM). As MPT 64 antigen is specific for MTBC and can be detected with considerable accuracy it can be used as a tool to differentiate between MTBC and NTM.

METHODS

200 samples (sputum, cerebrospinal fluid-CSF, pleural fluid etc.) in total were collected from clinically suspected smear negative patients. Each sample was inoculated both in L-J media and BACT / ALERT 3D system. Those samples which showed growth were further differentiated into MTBC and NTM both by conventional biochemical tests and MPT64 antigen detection kit test.

RESULTS

Out of the 200 samples, 30 produced growths. Lowenstein-Jensen (L-J) media detected 12 (40 %) and BACT / ALERT 3D system detected 26 (86.67 %) of the isolates. Among the 30 isolates, 18 (60 %) were MTBC and 12 (40 %) were NTM. Furthermore, L-J media detected 44.4 % and BACT / ALERT 3D system detected 88.9 % out of 18 MTBC isolates while among the 12 NTM isolates L-J media detected 33.33 % and BACT / ALERT 3D system detected 83.33 %. Mean detection time for MTBC was 46.5 days by L-J media and 20.8 days by BACT / ALERT 3D system. Mean detection time for NTM was 24.5 days by L-J media and 9.86 days by BACT / ALERT 3D system. Contamination rate in this study was 12 % in L-J media and 3 % in BACT / ALERT 3D system. We also found that for sensitivity, specificity, positive predictive value, negative predictive value of MPT64 antigen detection kit test was 100 % in our study considering biochemical tests as gold standard.

CONCLUSIONS

BACT / ALERT 3D system has a very good isolation rate and shorter mean detection time compared to L-J media even from smear negative samples. Also, MPT-64 antigen detection kit test is a very viable option to differentiate between MTBC and NTM.

KEYWORDS

MTBC, NTM, BACT / Alert 3D, MPT 64 Antigen

Corresponding Author:
Dr. Debalina Das,
8 B. L. Ganguly Lane,
Kolkata – 700033,
West Bengal, India.
E-mail: drdebalinadas63885@gmail.com

DOI: 10.18410/jebmh/2021/76

How to Cite This Article:
Sarkar R, Das D, Sarkar S. A study evaluating conventional and automated system of culture for isolation of mycobacteria from smear negative tuberculosis samples with special reference to MPT 64 antigen. J Evid Based Med Healthc 2021;8(07):391-395. DOI: 10.18410/jebmh/2021/76

Submission 14-09-2020,
Peer Review 20-09-2020,
Acceptance 26-12-2020,
Published 15-02-2021.

BACKGROUND

Tuberculosis (TB) has long been a global pandemic and India remains one of the major TB endemic countries. Further, the multi-drug resistant (MDR) tuberculosis & the TB–HIV-AIDS nexus are emerging as newer therapeutic problems.[1] The present recommendation of the Centers for Disease Control and Prevention to counteract this menace is to accomplish species identification of *M. tuberculosis* complex within 10 to 21 days after collection of specimens to expedite therapeutic intervention.[2] However, the identification of *Mycobacterium tuberculosis* complex isolates by conventional techniques is time consuming[1] and microscopy of samples stained with Ziehl–Neelsen staining dyes for acid fastness can detect only 30 % to 80 % of all specimens containing mycobacteria.[3] Moreover, culture on Lowenstein-Jensen solid medium, which still remains the "gold standard" for diagnosis of mycobacterial infection[4] rarely achieve these new CDC standards.[5]

Apart from the traditional microscopy and culture on L-J medium many systems have been developed in the recent years[6] like the new, automated liquid medium-based culture systems viz. BACT / ALERT 3D, BACTEC MGIT, Versa-TREK etc. These systems have led to a considerable shortening of detection time of mycobacteria as well as increased the sensitivity of isolation.

The BACT / ALERT 3D system is able to detect carbon dioxide released into the medium by actively growing mycobacteria by using a gas-permeable sensor. The sensor contains a colorimetric indicator embedded at the bottom of culture vials. The colour changes are monitored by a reflectometric detection unit contained inside each incubating drawer of the instrument. The final analysis is done by a computerized database management system.[7] So, in this study the efficiency of the automated BACT / ALERT 3D system will be evaluated in comparison to L-J medium with respect to estimation of recovery rate, mean detection rate and contamination time in smear negative samples.

Other than isolation of mycobacteria it is also clinically and therapeutically important to differentially identify *Mycobacterium tuberculosis* complex from nontuberculous mycobacteria. Such differentiation by routine laboratory method is time consuming and cumbersome. Most of the mycobacteriology laboratories use conventional biochemical tests to identify MTBC. These tests need special biosafety equipment whose procurement and installation are expensive.[8, 9] On the other hand, molecular methods that identify specific nucleic acid sequences of MTBC are rapid, sensitive and specific, but are expensive and require trained personnel and special laboratory setup.[10]

Hence, a rapid, accurate and simple test for characterisation of mycobacteria is required. Various biological, molecular and immunological studies of MTBC have resulted in identification of varieties antigens. Some of the antigens are specific to MTBC and nowadays used to differentiate between MTBC and NTM. MPT 64 is one such antigen. MPT 64, also termed as protein Rv 1980 c, is a protein secreted by actively growing MTB strains.[8,9] The MPT 64 antigen is absent in *M. bovis* BCG strains and *M. leprae* and in non-tubercular mycobacterial species. This has been confirmed by cloning and sequencing of *MPT 64* gene of H37 Rv culture filtrate.[11] The ongoing MTB research studies have proved that MPT64 have immunogenic property.[12,13]

The MPT 64 antigen, which is restricted to MTBC, can be detected with ease and substantial certainty by MPT 64 antigen detection kit.[1, 14] An immuno-chromatographic test (ICT) kit using mouse monoclonal antibodies against MPT64 antigen is being manufactured and marketed by commercial manufacturer and used to identify MTBC. So, it will be used in this study to identify MTBC and thus differentiate MTBC from NTM isolates.

Objectives

1. Evaluate whether BACT / ALERT 3D culture system provides any significant advantage over conventional culture methods for early diagnosis of mycobacterium species in smear negative sample;
2. Evaluate the efficacy of MPT 64 antigen kit for differentiation between *Mycobacterium tuberculosis* complex and non-tuberculous mycobacteria in culture positive sample.

METHODS

This cross-sectional study was undertaken between January - 2014 to December - 2014 in a tertiary care hospital after obtaining ethical clearance from the institutional ethics committee. 200 samples including sputum, and other smear-negative but clinically suspected pulmonary tuberculosis patients and extra-pulmonary tuberculosis patients who attended chest OPD or admitted in chest ward were selected for the study.

On the basis of an earlier study by Peirsimoni et al. who found recovery rate by BACT / ALERT 3D as 78.81 % and by L-J media 64.2 %, we calculated the sample size by using the formula:

$$\frac{(Z_\alpha + Z_\beta)^2 \times \{P_1(1-P_1) + P_2(1-P_2)\}}{d^2} = 146$$

Where Z_α = 1.96 at 95 % confidence interval, Z_β = 0.84 at power 80 %, P_1 = Proportion yield through BACT / ALERT 3D = 0.788, P_2 = Proportion yield through L-J media = 0.642, d = ($P_1 - P_2$). We found 146 by calculating with this formula. Assuming 20 % nonresponse rate final sample size was 176 (approximately). This was rounded to 200.

For the study we took total 200 sputum or other body fluid (CSF, pleural fluid, ascitic fluid etc.) samples appropriate for the disease. Then acid-fast bacilli (AFB) microscopy was done to rule out presence of AFB. We excluded the cases already diagnosed by sputum microscopy, or already diagnosed tuberculosis cases receiving treatment. After proper processing the samples were cultured in both automated BACT / ALERT 3D system and Lowenstein-Jensen (L-J) medium. All sample processing was done inside biosafety cabinet. Then mycobacteria (AFB) isolated from smear microscopy negative patient were further differentiated into MTBC and NTM on the basis of

conventional biochemical test (niacin test, nitrate reduction test, heat stable catalase test) and MPT64 antigen detection kit test.

Statistical Analysis

Is done by Excel spread sheet and OpenEpi platform. To find out association between yield of culture positive cases and culture methods chi-square test is used. To examine the difference in time to yield results between culture methods unpaired t test is used. P value < 0.05 was considered statistically significant.

RESULTS

In our study among 200 patient's majority were between 15 - 60 years of age (< 15 years 1 %, 15 – 60 years 81 %, > 60 years 18 %) while 68 % were males and 32 % were females. Out of 200 specimens 85 % were sputum and only 15 % were other (pleural fluid, CSF, ascetic fluid) samples. Among 30 (thirty) culture positive samples 12 (40 %) patients were grown in L-J media, 26 (86.67 %) patients were grown in BACT / ALERT 3D system and 8 patients were grown in both system (Table 1).

Culture System	Number of Growth of Mycobacterial Isolates	Percentage of Growth of Mycobacterial Isolates (%)
L-J	12	40
BACT / ALERT 3D	26	86.67
Table 1. Distribution of Growth of Mycobacterial Isolates in L-J Media and BACT / Alert 3D System (N = 30)		
Chi sq = 14.067; P value = 0.00017		

Mycobacteria (AFB) isolated from smear microscopy negative 30 patient were further differentiated into MTBC and NTM on the basis of conventional biochemical test (niacin test, nitrate reduction test, heat stable catalase test) and MPT64 antigen detection kit test. Out of 30 AFB isolated, 18 (60 %) were MTBC and 12 (40 %) were NTM. Considering biochemical tests as gold standard for sensitivity, specificity, positive predictive value, negative predictive value of MPT 64 antigen detection kit test was 100 % in our study.

$$\text{Sensitivity} = \frac{\text{TP}}{(\text{TP} + \text{FN})} = \frac{18}{(18 + 0)} = 100\ \%$$

$$\text{Specificity} = \frac{\text{TN}}{(\text{TN} + \text{FP})} = \frac{12}{(12 + 0)} = 100\ \%$$

$$\text{Positive Predictive Value} = \frac{\text{TP}}{(\text{TP} + \text{FP})} = \frac{18}{(18 + 0)} = 100\ \%$$

$$\text{Negative Predictive Value} = \frac{\text{TN}}{(\text{TN} + \text{FN})} = \frac{12}{(12 + 0)} = 100\ \%$$

In this study L-J media detected 8 (44.4 %) and BACT / ALERT 3D system detected 16 (88.9 %) out of 18 MTBC isolates (Table 2).

Culture System	Number of MTBC	Percentage of MTBC (%)
L-J	8	44.44
BACT / ALERT 3D	16	88.88
Table 2. Distribution of MTBC Isolated from L-J Media and BACT / Alert 3D System (N = 18)		
Chi sq = 8.000; P value = 0.0046		

We observed that L-J media detected 4 (33.33 %) and BACT / ALERT 3D system detected 10 (83.33 %) out of 12 NTM isolates (Table 3).

Culture System	Number of NTM	Percentage of Growth of NTM (%)
L-J media	4	33.33
BACT / ALERT 3D	10	83.33
Table 3. Distribution of NTM Isolated from L-J Media and BACT / Alert 3D System (N = 12)		
Chi sq = 6.171; P value = 0.013		

Mean detection time of MTBC by BACT / ALERT 3D system was less compared to L-J media. Mean detection time for MTBC in our study was 46.5 days and 20.8 days by L-J media and BACT / ALERT 3D system respectively (Table 4).

Culture System	Mean Detection Time (days)	Standard Deviation (SD)	Range of Detection Time in Days (2SD)
L-J media	46.5	5.75	35 to 56
BACT / Alert 3D system	20.8	4.07	12.67 to 28.94
Table 4. Comparison of Mean Detection Time of MTBC Isolated from L-J Media and BACT / Alert 3D System			
t = 12.71; P value < 0.0001			

Mean detection time of NTM isolates detected by BACT / ALERT 3D system was very fast compared to L-J media. Mean detection time for NTM in our study was 24.5 days and 9.86 days by L-J media and BACT / ALERT 3D system (Table 5).

Culture System	Mean Detection Time (Days)	Standard Deviation (SD)	Range of Detection Time in Days (2SD)
L-J media	24.5	1.75	21 to 28
BACT / Alert 3D system	9.86	1.415	7.03 to 12.69
Table 5. Comparison of Mean Detection Time of NTM Isolated from L-J Media and BACT / Alert 3D System			
t = 16.43; P value < 0.0001			

DISCUSSION

The study was conducted to evaluate the mycobacterial isolation by conventional Lowenstein-Jensen media and automated culture system that is BACT / ALERT 3D system from smear microscopy negative patients. Among 200 patient's majority were between 15 - 60 years of age (< 15 years 1 %, 15 – 60 years 81 %, > 60 years 18 %). Out of them 68 % were males and 32 % were females. Out of the specimens collected from these 200 patients 85 % were sputum and only 15 % were other (pleural fluid, CSF, ascetic

fluid) samples. Among these 200 samples only 30 grow in culture.

Among 30 (thirty) culture positive samples 12 (40 %) were grown in L-J media, 26 (86.67 %) were grown in BACT / Alert 3D system (Table 1) and 8 were grown in both systems. The difference in detection of mycobacterial isolates by each system is statistically significant (P-value = 0.00017). In a study by Piersimoni et al., found detection rates of clinically significant mycobacteria were 83.3 % for BACT / ALERT 3D and 69.7 % for L-J media.[7] Another study by Carricajo et al. found recovery rates for all mycobacteria were 94 % and 79 % for the BACT / ALERT 3D system and LJ medium respectively.[15] So our study is very much comparable to the study conducted by Piersimoni et al. and Carricajo et al. in case of detection by BACT / ALERT 3D system though detection by LJ medium is poor in our study compared to other studies.

Mycobacteria (AFB) isolated from smear microscopy negative 30 patient were further differentiated into MTBC and NTM on the basis of conventional biochemical test (niacin test, nitrate reduction test, heat stable catalase test) and MPT64 Antigen detection kit test. Out of 30 AFB isolated, 18 (60 %) were MTBC and 12 (40 %) were NTM which is almost very similar to that study of Maurya et al. where they showed that 67 % of mycobacterial isolates were MTBC and 33 % were NTM.[16] Considering biochemical tests as gold standard, the sensitivity, specificity, positive predictive value and negative predictive value obtained using MPT 64 antigen detection kit test was 100 % in our study. Arora J et al. from India in a study showed the sensitivity, specificity, positive predictive values, and negative predictive values of the MgMPT64 kit were 100, 96.4, 98.72, and 100 %, respectively.[17]

In this study L-J media detected 8 (44.4 %) and BACT / ALERT 3D system detected 16 (88.9 %) out of 18 MTBC isolates (Table 2). The difference was statistically significant (P-value = 0.0046). Peirsimoni et al. detected MTBC isolation rates of 91.3 and 78.3 by BACT / ALERT 3D system and L-J media respectively.[7] But in our study MTBC isolation by L-J media is poor compared to other studies.

We observed that L-J media detected 4 (33.33 %) and BACT / ALERT 3D system detected 10 (83.33 %) out of 12 NTM isolates (Table 3). This difference in detection is also statistically significant (P-value = 0.013). Our study could not be compared with other studies where NTM were detected from smear negative sample as any pertinent data was unavailable.

Mean detection time of MTBC by BACT / ALERT 3D system was less compared to L-J media. Mean detection time for MTBC in our study was 46.5 days and 20.8 days by L-J media and BACT / ALERT 3D system respectively (Table 4) and this difference was statistically significant (P-value < 0.0001). Martinez et al. from Cuba in their study found mean detection time for MTBC by BACT / ALERT 3D system to be 16.435 days while by L-J media the same was 33.577 days.[18] Peirsimoni et al. in their study found mean detection time as 32.1 days and 19.9 days by L-J media and BACT / ALERT 3D system respectively.[7] So the study by Martinez et al. and Peirsimoni et al. showed better result than our study in the issue of isolation of mycobacteria with less mean detection time by L-J media.

Mean detection time of NTM isolates detected by BACT / ALERT 3D system was very fast compared to L-J media. Mean detection time for NTM in our study was 24.5 days and 9.86 days by L-J media and BACT / ALERT 3D system (Table 5) respectively and this difference is also statistically significant (P-value < 0.0001). Whereas Martinez et al. found mean detection time for NTM as 35.952 days and 10.956 days by L-J media and BACT / ALERT 3D system respectively.[18] In case of detection of mean detection time for NTM by L-J media our study yielded significantly higher than that of Martinez et al.

Contamination rate in this study was 12 % in L-J media and 3 % in BACT / ALERT 3D system which was evidently quite lesser in comparison. Contamination rates in L-J media was 5.07 %, 3.08 %, 7.8 % and 10.1 % in studies by Lee et al, Uddin et al, Peirsimoni et al, and Martinez et al. respectively.[5,6,7,18] So, the contamination rate in L-J media was higher in our study. Contamination rate in BACT / ALERT 3D system was 3.62 %, 7.1 % and 4.6 % in studies done by Uddin et al, Peirsimoni et al. and Martinez et al. respectively.[6,7,18] So, the contamination rate of our study was very much comparable and slightly less than other studies.

CONCLUSIONS

BACT / ALERT 3D system is an automated suitable method for recovering tuberculous and non-tuberculous mycobacteria from various types of clinical samples. It has a very good recovery rate and shorter mean detection time compared to L-J media even from smear negative samples. So, by using BACT / ALERT 3D system diagnosis can be done, and treatment can be initiated within a short duration of time. This will reduce morbidity, mortality and prevent treatment failure and development of drug resistance. As few mycobacterial isolates may be missed by BACT / ALERT 3D system, maximum recovery can be achieved by combined use of BACT / ALERT 3D system and L-J media, which is also recommended by Centre for Disease Control and Prevention and World Health Organization.

MPT 64 antigen kit used in this study showed sensitivity, specificity positive predictive value and negative predictive value of 100 % when compared with biochemical methods of detection which are time consuming. Molecular methods of identification are rapid, sensitive and specific, but require trained personnel, expensive laboratory aids and are difficult to set up in resource constrained settings like India. Utilising the MPT 64 antigen detection kit may be a good alternative for detection of MTBC and differentiating MTBC from NTM among mycobacterial isolates and can be done within 15 minutes.

Data sharing statement provided by the authors is available with the full text of this article at jebmh.com.
Financial or other competing interests: None.
Disclosure forms provided by the authors are available with the full text of this article at jebmh.com.

REFERENCES

[1] Kumar VGS, Urs TA, Ranganath RR. MPT 64 antigen detection for rapid confirmation of M. tuberculosis isolates. BMC Research Notes 2011;4:79.

[2] Centers for Disease Control and Prevention. Essential components of a tuberculosis prevention and control program. Recommendations of the Advisory Council for the Elimination of Tuberculosis. Morbid Mortal Weekly Rep 1995;44(RR-11):1-16.

[3] Rohner P, Ninet B, Metral C, et al. Evaluation of the MB/BACT system and solid media for isolation of mycobacteria from comparison to the BACTEC 460 system and solid media for isolation of mycobacteria from clinical specimens. J Clin Microbiol 1997;35(12):3127-3131.

[4] Centers for Disease Control and Prevention. Updated guidelines for the use of nucleic acid amplification tests in the diagnosis of tuberculosis. MMWR Morbid Mortal Wkly Rep 2009;58(1):7-10.

[5] Lee JJ, Suo J, Lin CB, et al. Comparative evaluation of the BACTEC MGIT 960 system with solid medium for isolation of mycobacterium. Int J Tuberc Lung Dis 2003;7(6):569-574.

[6] Uddin MN, Uddin MJ, Mondol MEA, et al. Comparison of conventional and automated culture system for isolation of *Mycobacterium tuberculosis*. JAFMC Bangladesh 2009;5(1):14-17.

[7] Piersimoni C, Scarparo C, Callegaro A, et al. Comparison of MB/BACT ALERT 3D system with radiometric bactec system and löwenstein-jensen medium for recovery and identification of mycobacteria from clinical specimens: a multicenter study. J Clin Microbiol 2001;39(2):651-657.

[8] Wang Z, Potter MB, Gray MA, et al. The Solution structure of Antigen MPT64 from *Mycobacterium tuberculosis* defines a new family of beta-grasp proteins. J Mol Biol 2007;366(2):375-381.

[9] Yamaguchi R, Matsuo K, Yamazaki A, et al. Cloning and characterization of the gene for immunogenic protein MPB64 of Mycobacterium bovis BCG. Infection and Immunity 1989;57(1):283-288.

[10] Hasegawa N, Miura T, Ishii K, et al. New simple and rapid test for culture of *Mycobacterium tuberculosis* complex: a multi-centric study. J Clin Microbiol 2002;40(3):908-912.

[11] Gennaro ML. Immunologic diagnosis of tuberculosis. Clin Infect Dis 2000;30(Suppl 3):S243-246.

[12] Yu DH, Hu XD, Cai H. Efficient tuberculosis treatment in mice using chemotherapy and immunotherapy with the combined DNA vaccine encoding Ag85B, MPT-64 and MPT-83Efficient TB therapy with INH+PZA and combined DNA vaccine. Gene Therapy 2008;15(9):652-659.

[13] Harboe M, Nagai S, Patarroyo ME, et al. Properties of proteins MPB64, MPB70 and MPB80 of Mycobacterium bovis BCG. Infect Immun 1986;52(1):293-302.

[14] Fu R, Wang C, Shi C, et al. An improved whole-blood gamma interferon assay based the CFP21-MPT64 fusion protein. Clin Vaccine Immunol 2009;16(5):686-691.

[15] Carricajo A, Fonsale N, Vautrin AC, et al. Evaluation of BACT/Alert 3D liquid culture system for recovery of mycobacteria from clinical specimens using sodium dodecyl (Lauryl) Sulfate-NaOH decontamination. J Clin Microbiol 2001;39(10):3799-3800.

[16] Maurya AK, Nag VL, Kant S, et al. Evaluation of an immunochromatographic test for discrimination between *Mycobacterium tuberculosis* complex & non tuberculous mycobacteria in clinical isolates from extra-pulmonary tuberculosis. Indian J Med Res 2012;135(6):901-906.

[17] Arora J, Kumar G, Verma AK, et al. Utility of MPT64 antigen detection for rapid confirmation of *Mycobacterium tuberculosis* complex. J Glob Infect Dis 2015;7(2):66-69.

[18] Martinez MR, Sardiñas M, Garcia G, et al. Evaluation of BACT/ALERT 3D system for mycobacteria isolates. Journal of Tuberculosis Research 2014;2(2):59-64.