International Journal of Research in Medical Sciences
Gupta S et al. Int J Res Med Sci. 2016 Sep;4(9):3982-3987
www.msjonline.org
pISSN 2320-6071 | eISSN 2320-6012

DOI: http://dx.doi.org/10.18203/2320-6012.ijrms20162919

Research Article

Evaluation of various culture and staining techniques for the detection of extra pulmonary tuberculosis

Seema Gupta, Rahul K. Goyal, Rajesh Bareja*, Rabindra N. Behara

Department of Microbiology, SRMS Institute of Medical Sciences, Bareilly, Uttar Pradesh, India

Received: 02 July 2016
Accepted: 30 July 2016

***Correspondence:**
Dr. Rajesh Bareja,
E-mail: rajeshbareja@gmail.com

ABSTRACT

Background: Though pulmonary tuberculosis form is the commonest presentation, the extra pulmonary tuberculosis (EPTB) is also an important emerging clinical problem. The objective of the current study was to compare two staining techniques, Ziehl-Neelsen (ZN) stain, fluorescent stain and two-culture medium, solid Löwenstein-Jensen (LJ) medium and liquid 7H9 Middle brook medium in MGIT (Mycobacterium Growth Indicator Tube) 320 system, for detection of *Mycobacterium* in clinically suspected patients of EPTB.
Methods: A total of 100 clinically suspected cases of EPTB samples from various extrapulmonary sites had been collected. All the specimens were stained with ZN stain and fluorescent stain. The culture were processed after decontamination of specimens with NaOH-NALC method and thereafter inoculated on solid and liquid culture medium.
Results: Out of the 100 EPTB specimens, 30 were found positive by any of the above techniques used. Out of 30 positive cases 18 showed positivity by ZN staining while 20 showed positivity by fluorescent staining technique. In two culture methods, 27 isolates were grown by any of the culture system. Out of 27, 22 and 26 specimens showed growth of MTB complex on LJ media and MGIT culture system respectively. In AFB smear positive specimens, the average turnaround time was found to be 8.45 days and 22.5 days in MGIT and LJ medium culture assay respectively. While the turnaround times in AFB smear negative cases, it was 16.5 days and 32.3 days in MGIT and LJ medium culture assay respectively.
Conclusions: MGIT was a dependable, highly efficient system for recovery of MTB complex for EPTB specimens in combination with LJ media.

Keywords: EPTB, Fluorescent staining, LJ media, MGIT 320, ZN staining

INTRODUCTION

Tuberculosis (TB) is an infectious disease caused by the bacillus *Mycobacterium tuberculosis* is typically affects the lungs (pulmonary TB) but can affect other sites as well (extrapulmonary TB).[1] TB now ranks alongside HIV (Human Immunodeficiency Virus) as a leading cause of death worldwide. HIV's death toll in 2014 was estimated at 1.2 million, which included the 0.4 million TB deaths among HIV-positive people. Worldwide, 9.6 million people are estimated to have fallen ill with TB in 2014: 5.4 million men, 3.2 million women and 1.0 million children. Globally, 12% of the 9.6 million new TB cases in 2014 were HIV-positive.[1]

Studies involving immunocompetent adults have revealed that extra-pulmonary TB (EPTB) constituted about 15 to 20% of all cases of TB. With global rise of HIV infection, EPTB accounts for more than 50% of all cases of TB among HIV positive patients.[2] In developing countries the diagnosis of EPTB with conventional diagnostic tools is a major challenge. Smear for AFB

(acid fast bacilli) is reported to be positive in less than 10 to 37% of patients and mycobacterial culture is reported positive in variable proportion (12 to 80%) in various EPTB samples.[2] Although AFB microscopy and conventional Lowenstein Jensen (LJ) media culture assay remain the cornerstone for the diagnosis of TB, the sensitivity of these traditional methods is quite low, especially in the samples containing small number of organisms as is very common in EPTB cases.[3,4]

There is a need for rapid, sensitive and accurate detection of MTB (*Mycobacterium tuberculosis*) complex in clinical specimens to hasten the administration of appropriate antimycobacterial therapy and to prevent the spread of infection in the community. A variety of manual and automated systems have been developed specifically to reduce the time of detection and identification of *Mycobacteria* in clinical specimens. The objective of the current study was to compare MGIT (Mycobacterium Growth Indicator Tube) 320 with conventional LJ media culture assay, direct AFB smear examination by ZN (Ziehl-Neelsen) staining and fluorescent staining method and to diagnose mycobacterial infections from clinical specimens of EPTB cases.

METHODS

This cross-sectional study was carried out in a tertiary care hospital during February 2013 to January 2014. A total of 100 clinically suspected cases of extra pulmonary tuberculosis patients attending the Out Patient Departments and Indoor wards of various clinical departments (Medicine, Pulmonary tuberculosis and chest diseases, Obstetrics and gynaecology, Paediatrics, Orthopaedics) were included in the study.

The specimens were collected from patients of all age group and both sexes. The samples were found to be composed of pleural fluid (42), ascetic fluid (16), endometrial biopsy (12) including the sample of menstrual blood (1), cerebrospinal fluid (10), fine needle aspiration cytology of lymph nodes (10), pus (5), synovial fluid (4), pericardial fluid (1). Informed and written consent was taken from patients before enrollment. The ethical approval was obtained from institutional ethical committee to conduct the study.

Sample processing

All clinical specimens were processed for staining and culture using biological safety cabinet (BSL III) in mycobacterial laboratory. The sterile body fluids (pleural fluid, ascetic fluid, cerebrospinal fluid, FNAC lymph node, synovial fluid, and pericardial fluid) were concentrated by centrifugation in sterile container only before being inoculated. Pus and endometrial tissue specimens were digested and decontaminated by standard N-acetyl-L-cyteine-NaOH (NALC-NaOH) procedure.[4,5] After centrifugation, AFB smears were made for all clinical specimens studied using the sediment and stained with the Ziehl-Neelsen and fluorescent staining technique (Figure 1 and 2). The remaining sediment was suspended in 1-2 mL of sterile 0.67 M phosphate buffer (pH 6.8) and vortexed for 15 s. This suspension was used for inoculation of respective culture media. Liquid media was inoculated first, followed by the egg-based solid LJ media.[4]

The MGIT culture tubes and LJ media were inoculated with 0.5 mL suspension of the processed specimens. MGIT tubes were incubated inside the MGIT-320 instrument for 6 weeks according to manufacturer in-structions and LJ media was incubated for 8 weeks in the incubator at 37°C. MGIT-320 instrument detects the growth automatically, flashing red light to indicate instrument positive tubes and green for negative ones. The LJ media was checked twice weekly for first two weeks and then once every week for maximum period of 8 weeks.

The MGIT tubes and LJ slants (Figure 3) showing positive growth were further subjected to AFB smear and rapid card test for MTB complex, Bioline SD TB Ag MPT 64 Rapid (Standard Diagnostic, Inc., Republic of Korea).

For MGIT, the card test was done by according to manufacturer's instructions. After thorough mixing the suspension, a 100 µl of it is poured in to the sample well. After 15 minutes the appearance of red line in test 'T' line indicates the growth of MTB complex (Figure 4). For LJ slants, we make the suspension after emulsify 3-4 colonies in 200 µl of extraction buffer and use it as wide supra. The growth from MGIT tubes was also inoculated on blood agar to see the bacterial contamination. For LJ slant, bacterial contamination was detected by performing Gram staining from the suspected colonies. A reference strain H37Rv was used for quality control.

RESULTS

Out of 100 EPTB specimens, MTB complex was found to be positive in 30 cases by any of the above techniques i.e. AFB staining, fluorescent staining, LJ media and MGIT culture system. A total of 13 samples were positive by all the methods.

By staining techniques, out of 30 positive cases, 18 samples were found to be positive by both ZN staining and fluorescent staining while 18 showed positivity by ZN staining and 20 samples showed positivity by fluorescent staining respectively (Table 1 and 2). Three cases were smear positive but did not show any growth by any culture methods. A total of 27 specimens were found to be positive by any of the culture methods. Out of 27 positive specimens, 21 were positive by both culture methods. Twentytwo specimens showed growth of *Mycobacterium* on LJ media while one specimen was positive with LJ culture but negative by MGIT culture

assay. On the other hand, in MGIT 320, *Mycobacterium* was isolated in 26 specimens out of which five specimens showed growth only on MGIT but were found negative by LJ media culture assay (Table 1 and 3). MGIT culture assay system was found to be more sensitive in detection of *Mycobacterium* i.e. 96.29% (26/27) cases with average detection time of 12.45 days. In LJ medium culture assay the positivity was 87.18% (22/27) with average detection time of 27.4 days (Table 4).

Table 1: Comparison among various techniques for detection of MTB complex in various EPTB specimens.

Specimens	Total no. (n=100)	ZN staining positive	Fluorescent staining positive	Culture LJ media positive	Culture MGIT 320 positive
Pleural fluid	42	5 (11.9%)	5 (11.9%)	8 (19%)	11 (26.9)
Ascitic fluid	16	1 (6.25%)	1 (6.25)	2 (12.2%)	3 (18.7%)
Endometrial biopsy	12	2 (16.5%)	3 (25%)	2 (16.5%)	3 (25%)
Cerebrospinal fluid	10	2 (20%)	2 (20%)	1 (10%)	1 (10%)
FNAC lymph node	10	6 (60%)	7 (70%)	6 (60%)	5 (50%)
Pus	5	2 (40%)	2 (40%)	2 (40%)	2 (40%)
Synovial fluid	4	0 (0%)	0 (0%)	1 (25%)	1 (25%)
Pericardial fluid	1	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Total samples	100	18 (18%)	20 (20%)	22 (22%)	26 (26%)

Table 2: Cross tabulation of fluorescent staining with ZN staining.

		ZN staining		Total
		AFB seen	No AFB seen	
Fluorescent staining	AFB seen	18	2	20
	No AFB seen	0	80	80
		18	82	100

Regarding AFB smear positive specimens, the average turnaround time was found to be 8.45 days and in smear negative cases it was 16.5 days in MGIT culture system. The average turnaround time taken by MGIT culture system was 12.4 days. While the turnaround time by LJ medium culture assay in smear positive cases was 22.5 days and in smear negative cases it was 32.3 days. The average turnaround time for LJ medium culture assay was 27.4 days.

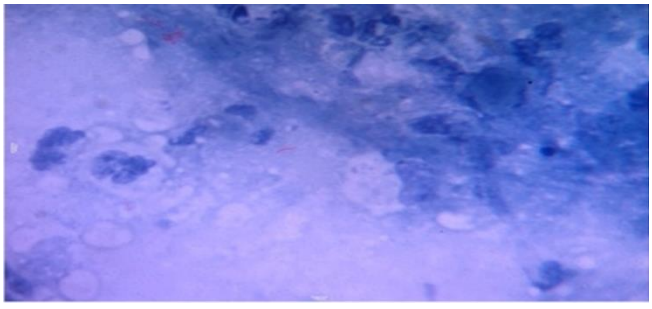

Figure 1: Acid fast bacilli in Ziehl Neelsen stained smear (100x).

By comparing two staining methods, fluorescent staining and ZN staining, the sensitivity of fluorescent staining was found to be 100% while specificity 97.5%. The positive predictive value was found to be 90% and negative predictive value was found to be 100%. The diagnostic accuracy was found to be 98%. By comparing two culture methods, the sensitivity of MGIT was found to be 95.4% and specificity was found to be 93.4%.

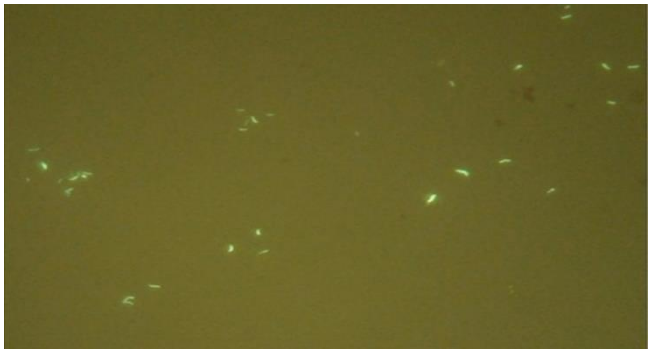

Figure 2: Fluorescent dye stained smear of tubercle bacilli under LED fluorescent microscope (40X).

The positive predictive value of MGIT was found to be 80.8% and negative predictive value was found to be 98.6%. The diagnostic accuracy was found to be 94%. Besides better sensitivity and specificity the turnaround time of MGIT was found less than LJ culture media assay. For staining and culture, ZN staining and LJ media culture method were taken as the gold standard.

Figure 3: Growth of *M. Tuberculosis* on Lowenstein-Jensen medium.

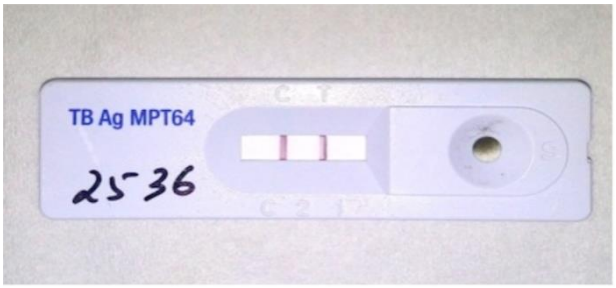

Figure 4: TB Ag MPT 64 card test showing the positive test line 'T' for MTB complex.

Table 3: Cross tabulation of MGIT with LJ media.

		LJ Culture Media		Total
		Growth	No Growth	
MGIT320 Media	Growth	21	5	26
	No Growth	1	73	74
		22	78	100

Table 4: Culture positivity and turnaround time of various EPTB specimens.

Samples	AFB smear ZN staining	No. of samples	MGIT320 positive	Turnaround time (range in days)	LJ culture Positive	Turnaround time (range in days)
Pleural fluid	Positive	5	5	9	5	22
	Negative	37	6	14	3	30
Ascitic fluid	Positive	1	1	10	1	23
	Negative	15	1	17	1	32
Endometrial biopsy	Positive	2	2	10	1	25
	Negative	10	1	NA	0	NA
Cerebrospinal fluid	Positive	2	1	9	6	21
	Negative	8	0	NA	0	NA
FNAC Lymph node	Positive	6	5	8	2	23
	Negative	4	0	19	0	NA
Pus	Positive	2	2	8	2	21
	Negative	3	0	NA	0	NA
Synovial fluid	Positive	0	0	NA	0	NA
	Negative	4	1	16	1	35
Pericardial fluid	Positive	0	0	NA	0	NA
	Negative	1	0	NA	0	NA

DISCUSSION

TB mortality has fallen 47% since 1990, with nearly all of that improvement taking place since 2000, when the MDGs [Millennium Development Goals (MDGs)] were set. In all, effective diagnosis and treatment of TB saved an estimated 43 million lives between 2000 and 2014.[1]

In the present study, out of 100 samples, 30 specimens were positive by any of the methods. On consideration of staining methods, 20 specimens were smearing positive by fluorescent staining and 18 samples were positive with ZN staining technique. The detection rate by fluorescent staining was 66.7% (20/30) while by ZN staining it was 60% (18/30).

These results showed that fluorescent staining technique is more sensitive in detection of AFB in EPTB samples as compared to ZN staining. These results are concordant with various studies done by Githui et al (80% by fluorescent staining, 65% by ZN staining), Ulukanligil et al (85.2% by fluorescent staining, 67.6% by ZN staining), Murray et al (93% by fluorescent staining, 73% by ZN staining), Prashanthi et al (69% by fluorescent staining, 50% by ZN staining) and Jain et al (41% by fluorescent

staining, 32% by ZN staining).[6-10] The use of fluorescent stain greatly improves the diagnostic value of the EPTB smears with a low density of bacilli (Paucibacillary) which are likely to be missed on ZN stained smear. This study showed the higher isolation rate of MTB complex in MGIT culture system (96.29%) than the conventional LJ medium culture assay (81.48%). Rishi et al observed 98.6% isolation rate by MGIT and 63.95% by LJ medium culture assay.[11]

Various authors have also reported the similar findings ranging from 80 to 100% for MGIT and 59.7 to 87.2% for LJ medium culture assay.[12-14] In the current study, isolation rate of MGIT culture system was 14.81% more than the LJ medium culture assay. Besides higher isolation rate, even the time to detect *Mycobacterium* was shorter on MGIT than on LJ medium culture assay (Table 2). Three cases were smear positive but did not show any growth by any culture methods. The possible reason for no growth could be the absence of live organism from the particular specimen. In this study, one sample was culture negative with MGIT but showed growth in LJ culture medium assay. This was due to the contamination in MGIT culture tube that showed the growth of contaminant bacteria on blood agar plate.

In the current study, the time to detection of mycobacteria was shorter in smear positive EPTB specimens. In smear positive specimens, growth of mycobacteria was detected in average 8.45 days (7 to 15 days) with MGIT as compared to LJ medium assay (22.5 days). While in smear negative specimens, average time to detection of mycobacteria was 16.5 days (9-22days) with MGIT as compared to LJ medium assay (32.3 days). Some authors also reported the similar observations.[13-18]

The present study showed the contamination rate of 8% with MGIT and of 11% with LJ media culture mehod which correspond to various other studies that showed 3.7% to 10% with MGIT and 1.2 to 21.1% with LJ method.[11,14,17] The possible reason for the significant reduction in the contamination rate could be the use of the N-acetyl cysteine sodium hydroxide (NALC-NaOH) method and the addition of the MGIT-PANTA mixture.

CONCLUSION

The current study concludes that MGIT has been proven as a dependable, efficient system for recovery of *M. tuberculosis* complexes from paucibacillary extra-pulmonary TB specimens when used in combination with LJ media. This would hasten the administration of appropriate antimycobacterial therapy thereby decreasing morbidity and mortality as well as preventing the spread of infection in the community.

Funding: No funding sources
Conflict of interest: None declared
Ethical approval: The study was approved by the Institutional Ethics Committee

REFERENCES

1. WHO global tuberculosis report 2015. www.who.int/tb/publications/global_report/en/.
2. Sharma SK, Mohan A. Extrapulmmonary tuberculosis-Review article. Indian J Med Res. 2004;120:316-53.
3. Negi SS, Khan SF, Gupta S, Pasha ST, Khare S, Lal S. Comparison of conventional diagnostic modalities, BACTEC culture and PCR test for diagnosis of tuberculosis. Indian J Med Microbiol. 2005;23:29-33.
4. Pottathil S, Nair A, Singh V, Kumar S, Bareja R. Evaluation of rapid techniques for the detection of mycobacteria in sputum with scanty bacilli or clinically evident, smear negative cases of pulmonary and extra-pulmonary tuberculosis. Mem Inst Oswaldo Cruz. 2011; 106 (5): 620-24.
5. Kent PT, Kubica GP. 1985. Public health mycobacteriology - a guide for the level III laboratory, Department of Health and Human Services/Centers for Disease Control and Prevention, Atlanta. 207.
6. Githui W, Kitui F, Juma ES, Obwana DO, Mwai J, Kwamasga D. A comparative study on reliability of the fluorescent microscopy and Ziehl Neelsen method in the diagnosis of pulmonary tuberculosis. East Afr Med J. 1993;70:263-6.
7. Ulukanligil M, Aslan G, Tasci S. A comparative study on the different staining methods and number of specimens for the detection of acid fast bacilli. Mem Inst. Oswaldo Cruz. 2000;95:855-8.
8. Murray SJ, Barrett A, Magee JG, Freeman R. Optimisation of acid fast smears for the direct detection of Mycobacteria in clinical samples. J Clin Path. 2003;56:613-5.
9. Prasthani K, Kumari AR. Efficacy of fluorochrome stain in the diagnosis of pulmonary tuberculosis coinfected with HIV. Ind J Med Microbiol. 2005;23(3):179-85.
10. Jain A, Bhargawa A, Agarawal SK. A comparative study of two commonly used staining techniques for acid fast bacilli in clinical specimens. Ind J Tub. 2002;49:161.
11. Rishi S, Sinha P, Malhotra B. A comparative study for the detection of Mycobacterium by BACTEC MGIT 960, Lowenstein Jensen media and direct AFB smear examination. Ind J Med Microbiol. 2007;25(4);383-6.
12. Lu D, Heeren B, Dunne WM. Comparison of the automated MGIT with Lowenstein Jensen media for recovery of Mycobacteria from clinical specimens. Am J Clin Pathol. 2002;118:542-45.
13. Hanna BA, Ebrahimzadeh A, Elliot LB, Morgan MA, Novak SM, Rusch-Gerdes S, et al. Multicentre evaluation of BACTEC MGIT960 system for recovery of Mycobacteria. J Clin Microbiol. 1999;37:748-52.
14. Alcaide F, Benitez MA, Escriba JM, Martin R. Evaluation of BACTEC MGIT 960 and the

MB/BacT systems for recovery of Mycobacteria from clinical specimens and for species identification by DNA Accu Probe. J Clin Microbiol 2000;38:398-01.
15. Tortoli E, Cichero P, Pietsimoni C, Simonetti MT, Gesu G, Nista D. Use of BACTEC MGIT 960 for recovery of Mycobacteria from clinical specimens:Multicentre study. J Clin Microbiol. 1999;17:578-82.
16. Idiogras P, Beristain X, Iturzaeta A, Vicente D, Perez Trallero E. Comparison of the automated non radiometric BACTEC MGIT 960 system with Lowenstein Jensen media. Colestos, MB 7H11 solid media for recovery of Mycobacteria. Eur J Clin Microbiol Infect Dis. 2000;19:350-54.
17. Somoskovi A, Kodman C, Lantos A, Bartfai Z, Tamasi L, Fuzi J, et al. Comparison of recoveries of Mycobacterium tuberculosis using the automated BACTEC MGIT960 system, BACTEC 460 TB system and Lowenstein Jensen media. J Clin Microbiol. 2000;38:2395-397.
18. Huang TS, Chen CS, Lee SS, Huang WK, Liu YC. Comparison of BACTEC MGIT960 and BACTEC460 TB system for the detection of Mycobacteria in clinical specimens. Am Clin Lab Sci. 2001;31:279-83.

Cite this article as: Gupta S, Goyal RK, Bareja R, Behara RN. Evaluation of various culture and staining techniques for the detection of extra pulmonary tuberculosis. Int J Res Med Sci 2016;4: 3982-7.