Original Article

Evaluation of multiple laboratory methods in the diagnosis of extrapulmonary tuberculosis

Abstract

Introduction: Tuberculosis remains a worldwide public health problem. The emergence of human immunodeficiency virus infections (HIV) has further complicated the disease burden as it has rapidly increased the risk of pulmonary and extrapulmonary tuberculosis (EPTB). EPTB is an important clinical entity. The precise diagnosis is very important because early detection of cases and effective treatment if instituted at the right time completely cures the patients of the disease. **Objectives:** To find out sensitivity and specificity of Ziehl-Neelsens staining (ZN), Auramine staining, and rapid slide culture technique (RSC), comparing them with growth on Lowenstein-Jensens medium (ZN) as the gold standard. **Materials and Methods:** The present study included 66 clinical specimens from patients suspected of EPTB inclusive of HIV-infected patients. They were subjected to ZN and Fluorescent method of staining, culturing by LJ and RSC method, and comparing growth on LJ medium as the gold standard. **Results and Conclusion:** *Mycobacterium tuberculosis* was isolated in 24.2%. Nontuberculosis *Mycobacterium* was isolated in 6% of the 66 samples processed. The highest number of isolation was from lymph node aspirates (83.3%). 2% of the samples were HIV seropositive.

Key words: Extrapulmonary tuberculosis, Lowenstein-Jensens, rapid slide culture

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INTRODUCTION

Tuberculosis remains a worldwide public health problem even after 100 years of discovery of *Mycobacterium tuberculosis*.[1] Approximately 9 million people develop acute tuberculosis every year worldwide.[2] The emergence of human immunodeficiency virus (HIV) infection has further complicated the disease burden as it has rapidly increased the risk of tuberculosis.

Tuberculosis can involve any organ system in the body while the pulmonary tuberculosis (PTB) is the most common presentation. The term extrapulmonary tuberculosis (EPTB) has been used to describe isolated occurrences of tuberculosis at body sites other than the lungs.[3] EPTB constitutes about 15-20% of all cases of tuberculosis.[3] The clinical presentation of EPTB is atypical. They may present with pyrexia of unknown origin, and this may be the only clinical presentation.

The diagnosis of EPTB is very important because early detection of cases and effective treatment instituted at the appropriate time completely cures the patient. The increase in the incidence of HIV infections has further increased the incidence of EPTB,[4] it is found that among the HIV-TB co-infected patients, EPTB is about 30-70% more common than PTB.[4] There is no specific, sensitive, inexpensive, and rapid method of diagnosis. Tissue samples for the confirmation of diagnosis can sometimes be difficult to procure. EPTB often presents a diagnostic challenge, most often leading to delay in diagnosis.[5] The study is undertaken to see if different methods of diagnosis can improve the sensitivity of diagnosis of EPTB. Recently, there is renewed interest in rapid slide culture (RSC)[6] which was first used by Sir Robert Koch, though culture on Lowenstein-Jensens (LJ) medium remains the gold standard. RSC is useful for early detection of viable *M. tuberculosis* as the growth occurs within a period of 7 days.[7]

Objectives

1. To find out the sensitivity and specificity of Ziehl-Neelsen's (ZN) staining, Auramine. Staining, and RSC technique and comparing them with growth on LJ medium as the gold standard.

MATERIALS AND METHODS

66 clinical specimens which included pleural fluid, pus samples, lymph node aspirates, biopsy specimens, cerebrospinal fluid, synovial fluid, ascitic fluid, urine, and bone marrow aspirates from patients suspected of EPTB at RL Jalappa Hospital and Research Center were collected during the period of December 2008 to August 2010. Patients whose sputum was positive for acid fast bacilli (AFB) were excluded from the study. The specimens collected were sent to the laboratory immediately and were processed, in case of delay these specimens were kept at 4°C in the refrigerator after obtaining informed consent. These specimens were examined by ZN and fluorescent staining (Auramine staining) technique.[7] The samples were further concentrated by modified petroffs method and were cultured on LJ medium[8] and RSC method according to Nair *et al*.[6]

Human blood medium was used for RSC technique.[6] Unused but not >4 weeks old citrated human blood was used to prepare the human blood medium (HBM). The blood is diluted with equal volumes of sterile deionized water to cause hemolysis. The medium was made selective by adding trimethoprim (10 mg/l), amphotericin b (10 mg/l) and ceftazidime (100 mg/l). PH of the medium was adjusted between 6.5 and 7.5. Seven ml of this solution was dispensed in sterile screw capped McCartney bottles with antifungals and antibiotics to eliminate chances of contamination; this constituted one unit of HBM.

Smear was made on the lower one-third of a clean slide and air-dried. The slide was then immersed in the HBM in such way that smear on the slide remained dipped in the medium. Inoculation was done in duplicates. The bottle was incubated at 37°C for 7 days. On the 7th day slide was taken out, washed with distilled water and placed in an oven at 80°C for 30 min. Any growth was confirmed by ZN staining and microscopy under oil immersion objective for microcolonies of AFB. A known *M. tuberculosis* strain H37Rv was used as a positive control and an uninoculated slide as a negative control simultaneously.

Grading of culture by RSC method [Table 1].

The presence of micro colonies was demonstrated by ZN staining. Growth was graded according to the size of micro colonies [Figure 2].

The growth if any at the end of 12 weeks on LJ culture and by 7 days on RSC was confirmed by Niacin test. Growth on LJ culture [Figure 1] was considered as the gold standard.

RESULTS

In the present study, a total of 66 patients with clinical suspicion of EPTB was enrolled. 38 (57.5%) were males and 28 (42.5%) were females. Majority belonged to the age group of 21-30 years. Most of the samples were obtained from cases of pleural effusion (43.9%) followed by pus (16.6%), from parietal wall abscess, scrotal ulcer, psoas abscess, port site abscess following cholecystectomy, breast abscess, chronic suppurative otitis media, and lymph node aspirates (9.1%).

Of the 66 specimens processed, 22 were positive for AFB either by ZN stain, Auramine staining, LJ culture or RSC. 16 were identified as *M. tuberculosis* [Table 2] and 4 were nontuberculous *Mycobacterium* which included *Mycobacterium chelonae* and *Mycobacterium fortuitum* by culture and two were positive by staining methods only. Majority of the isolates were from lymph node aspirates 5 (83.3%). The percentage of positivity with ZN and fluorescent staining technique was 27.2%. The isolation rate by LJ culture was 30.4%, and RSC was 24.2%. 2 (3%) samples which were positive by ZN staining, fluorescent staining methods, but were negative by LJ culture and RSC.

Table 1: Grading of RSC method[16]

ZN staining	Grading
No multiplication of AFB as compared with an un-incubated control	0
Small clumps of up to four bacilli	1+
Large clumps of bacilli, but no cord formation	2+
Microcolonies with some cord formation	3+
Large micro colonies with good cord formation	4+

RSC = Rapid slide culture, ZN = Ziehl-Neelsens, AFB = Acid fast bacilli

Table 2: Isolation of *Mycobacterium tuberculosis* from different sites

Sample	Number of sample processed	Total positive (%)	Percentage
Lymph node aspirate	6	5	83.3
Pleural fluid	29	4	13.8
Pus	11	3	27.8
Synovial fluid	3	1	33.3
Urine	2	1	50.0
Ascitic fluid	3	1	33.3
Biopsy specimens	6	1	16.7
CSF	4	0	0.0
Bone marrow aspirate	2	0	0.0
Total	66	16 (24.2)	

CSF = Cerebrospinal fluid

Figure 1: Growth of Mycobacterium tuberculosis on lowenstein-Jensens medium

DISCUSSION

In the present study, there was male preponderance, with a maximum number of patients in the age group of 21-30 years. Our study showed a positivity of 22 (33.3%) by one or more methods of staining and culture. Narang *et al.*[9] showed a positivity of 35.94% considering staining technique by ZN method, LJ culture and histopathological examination in lymph node specimens only. Maximum number of isolation of *M. tuberculosis* was from lymph node aspirates (83.3%). Vanajakumar *et al.*[10] has reported 98% isolation from lymph node specimens. There was no significant difference observed between the results obtained by ZN and fluorescent method of staining. However, fluorescent staining has offered the advantage of screening the smears under low power where large numbers of slides are screened in less time reducing observers fatigue as quoted in the bulletin of ICMR.[11] RSC had sensitivity of 80%, specificity of 100%, and positive predictive value (PPV) of 100%, and negative predictive value of 92% [Table 3]. It had sensitivity comparable with smear microscopy. It has the advantage of demonstrating microcolonies by light microscopy in 7 days.

To the best of our search to date, there are only three studies on RSC technique from India. Purohit *et al.*[12] evaluated a new medium sheep blood medium and HBM and found that the results between the two were equally good.[12]

The newer methods in the detection of *M. tuberculosis* are Bactec system, mycobacterial growth indicator tube, and molecular methods which are rapid and effective. These tests are very expensive and technically demanding and beyond the reach of many laboratories in India. One important issue of concern in India is affordability, as we have a larger population who are poor.[13]

Rapid slide culture, when compared to the above methods, is rapid, simple, and affordable and the turnaround time for detection of *M. tuberculosis* is 7 days which proves it being more advantageous than LJ culture.[14]

Jena *et al.*[15] in 1995 and George *et al.*[16] in 1998 used HBM for drug susceptibility testing in pulmonary samples. Jena *et al.*[15] compared it with conventional LJ culture in fresh untreated cases of PTB. A positivity 0f 65.2% for RSC and 85.1% for LJ culture was shown in their study.[15]

In our study, we used the RSC method as a novel method for isolation of *M. tuberculosis* in extra-pulmonary specimens. The isolation rate was 24.2% in RSC and 30.4% in LJ culture showing slightly higher percentage of isolation on LJ culture. Jena *et al.* found RSC to be more sensitive than smear microscopy.[15] Nair *et al.*[6] found smear microscopy to be more sensitive than RSC. In our study, RSC had a sensitivity of 80% comparable with the sensitivity of smear microscopy. A PPV of 100% indicates that the diagnostic potential of the test is good.

It was found that 4 (6%) of the specimens were smear negative and culture positive [Table 4]. EPTB is paucibacillary in nature and hence most often they are not detected by smear microscopy. This can be explained because of less number of organisms present in the sample as, there must be at least 10^4 organisms in the sample to be detected by smear microscopy whereas the number of organisms required for culture to be positive is 10-100 organisms per ml.[17,18]

In the present study, 2 (3%) were smear positive and culture negative [Table 5]. Aparna *et al.* have shown 12.1% to be smear positive and culture negative in lymph node aspirates.[19]

This could be attributed to treatment with anti-tubercular drugs and broad spectrum antibiotics such as amoxicillin, fluoroquinolones reported to be inhibitory to *M. tuberculosis* that might lead to negative culture and positive smear. Further clinical history revealed that they were treated with levofloxacin for 2 weeks.

CONCLUSION

From the above findings, it can be concluded that RSC is as good as LJ culture. It is a rapid, cheap, and effective method for obtaining culture confirmation of tuberculosis and considered most suitable in a country like India where tuberculosis is rampant. The need of the hour being rapid detection of *M. tuberculosis* and prompt treatment. However, LJ medium still remains the gold standard.

Table 3: A comparison of sensitivity, specificity, PPV, NPV in different methods with LJ as the gold standard

Methods	Sensitivity	Specificity	PPV	NPV
Direct smear	80	95.65	88.89	91.67
Fluorescent stain	80	95.65	88.89	91.67
RSC	80	100	100	92

PPV = Positive predictive value, NPV = Negative predictive value, RSC = Rapid slide culture, LJ = Lowenstein–Jensens

Table 4: Specimens which were smear negative and culture positive

Specimens	Staining methods		Culture methods	
	ZN	Fluorescent	LJ	RSC
Pleural fluid	Negative	Negative	Positive	Positive
Ascitic fluid	Negative	Negative	Positive	Positive
Endometrial biopsy	Negative	Negative	Positive	Positive
Pus shoulder joint	Negative	Negative	Positive	Positive

RSC = Rapid slide culture, LJ = Lowenstein-Jensens, ZN = Ziehl-Neelsens

Table 5: Specimens which were smear positive and culture negative

Specimen	Staining methods		Culture methods	
	ZN	Fluorescent	LJ	RSC
Bone marrow aspirate	Positive	Positive	Negative	Negative
FNAC ileocecal region	Positive	Positive	Negative	Negative

RSC = Rapid slide culture, LJ = Lowenstein-Jensens, ZN = Ziehl-Neelsens, FNAC = Fine needle aspiration cytology

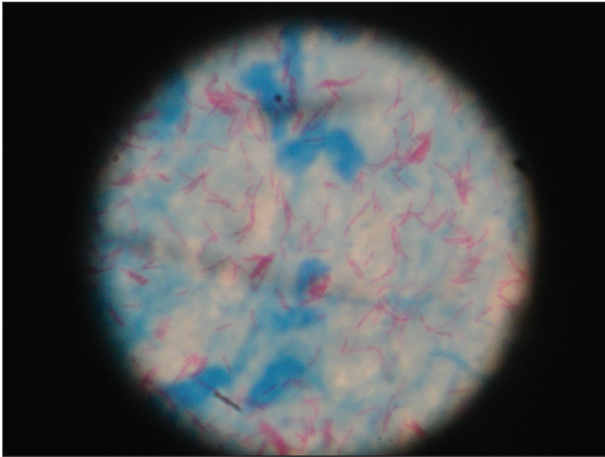

Figure 2: Zeil-Neelsens staining: Rapid slide culture showing microcolonies with some cord formation (grade-3+)

In the present study, HBM was used in RSC, blood was screened for all the pathogens associated with biohazard. However, an alternative like fetal calf serum can replace blood, and RSC can be used as a diagnostic test. It is useful in early confirmation of *M. tuberculosis* which makes it an ideal diagnostic test in a country like India where tuberculosis is rampant. However, since the sample size is small, a better conclusion can be derived by conducting a study with a larger number of samples.

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