ORIGINAL ARTICLE

Comparative study of Smear Microscopy, Rapid Slide Culture, and Lowenstein - Jensen culture in cases of pulmonary tuberculosis in a tertiary care hospital

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Background: Tuberculosis (TB), a dreadful disease known to mankind continues to be a problem in a developing country like India. The incidence of people getting infected with TB is on the rise due to compounding factors like coinfection with the human immunodefiency virus and multidrug-resistant strains. There is a definitive need for early diagnosis and treatment of TB to curb transmission of the infection. Direct smear microscopy, though cheap and rapid, lacks sensitivity. Isolation of *Mycobacterium tuberculosis* in culture requires a long time, because of which there is a need for a rapid method which has good sensitivity and specificity for the detection of *M. tuberculosis*. The present study was undertaken to determine the test which diagnoses TB rapidly and to compare the sensitivity of smear microscopy, concentration method, rapid slide culture, and Lowenstein - Jensen (LJ) culture. **Materials and Methods:** Sputum samples of 200 patients were subjected to direct smear and concentration by modified Petroff's method. The concentrated sputum was also taken for slide culture using human blood medium and inoculated on LJ media. **Results:** LJ culture was positive in 47 (23.5%) cases, of which three were nontubercular mycobacteria. Using LJ culture as the standard method, the sensitivity of direct smear, concentration method, and rapid slide culture method was 68, 83, and 89%, respectively, and specificity was 100% in all the three tests. **Conclusion:** Rapid slide culture showed good sensitivity which was comparable to and next in efficacy to LJ culture and this technique can be adopted in the Revised National Tuberculosis Control Program (RNTCP) as it is a rapid, cheap, sensitive, and specific method.

Key words: Direct microscopy, Lowenstein - Jensen media, pulmonary tuberculosis, rapid slide culture

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INTRODUCTION

Tuberculosis (TB), one of the ancient diseases known to affect mankind is considered to be a major cause of death among the infectious diseases prevalent in India.[1] Prompt detection, isolation, identification, and susceptibility testing of *Mycobacterium tuberculosis* from clinical samples are essential for appropriate management of patients with TB.[2]

Bacteriological examination of the clinical specimens plays an important role in the diagnosis of any mycobacterial infection, more so for TB. The widely used acid-fast smear (Ziehl Neelsen stain) for the demonstration of the acid-fast bacilli, though rapid and simple to perform, has a low sensitivity, especially for single and paucibacillary specimens. Also, it cannot differentiate live bacilli from inactive or dead bacilli. A definitive diagnosis of active mycobacterial infection therefore depends on the isolation and identification of mycobacteria from the clinical specimen, by culture. Traditional or conventional methods for mycobacterial culture utilize media containing egg or potato base (Middlebrook 7H10 or 7H11) or albumin (Lowenstein - Jensen medium, LJ). Although these media support the growth of mycobacteria, several weeks (2-8 weeks) of incubation maybe necessary before the growth can be detected. This duration may further be prolonged in the case of paucibacillary specimens.

There is thus a definite need for alternative culture methods that would rapidly detect and identify mycobacteria, including their drug-susceptibility patterns, from clinical specimens.[3]

Direct microscopy, though rapid and specific, requires 10,000 bacilli/mL in the specimen to be detected. Laboratory culture can be done which is considered

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as the gold standard test, but the recovery period of *M. tuberculosis* is long (6-8 weeks).[4]

Dickinson and Mitchison described a new slide culture technique that was rapid, simple, and safe but required the use of a fluorescent microscope. We have modified the above technique to obtain similar results with the use of a bright field microscope.[5]

Rapid slide culture is a sensitive, economical, and rapid method for diagnosis. This method uses human blood medium and an incubation period of seven days. The method was successfully followed by Jena *et al*.[6] This background study aimed to compare the sensitivity and specificity of microscopy, rapid slide culture, and LJ culture in clinically suspected cases of pulmonary TB.

MATERIALS AND METHODS

The study was conducted on 200 sputum samples obtained from clinically suspected and/or radiologically evident cases of pulmonary TB who were not on antitubercular drugs. Patients visiting, on an inpatient or outpatient basis, the Medicine and Pediatrics Department, Kempegowda Institute of Medical Sciences and Research Institute, Bangalore between August 2009 and August 2010 were included in the study.

Methodology

A sputum sample was collected from persons with a cough for two weeks or more, with or without other symptoms suggestive of TB. The samples were subjected to smear microscopy, concentration method, LJ culture, and modified slide culture for acid-fast bacilli.[7]

Zeihl-Neelsen staining

The smear was prepared from the purulent portion of sputum on a clean slide on an area of 2 × 1 cm. The smear was flooded with 1% concentrated carbol-fuchsin and heated from below till vapors were seen. Heating was repeated after an interval of 2½ minutes. The slide was washed with tap water. It was decolorized with 25% sulfuric acid for 2-4 minutes and then washed with tap water. The procedure was repeated. The slide was counterstained with 0.1% methylene blue for 10 seconds and washed with tap water once again. It was blotted with clean blotting paper and dried in air and observed under oil immersion lens.[8]

Modified petroff's method

This technique was used for decontamination of the sputum sample. Four milliliter of sputum was taken in a screw-capped bottle and an equal volume of 4% sodium hydroxide was added to it. The mixture was homogenized by shaking on a vortex machine for 15 minutes and then incubated at 37°C for 20 minutes. The mixture was centrifuged at 3,000 rpm, for 15 minutes. The supernatant was poured off in a disinfectant solution and the deposit was resuspended in 15 mL of sterile distilled water. The solution was centrifuged once again at 3,000 rpm for 15 minutes. The supernatant fluid was poured off and the deposit was used for further processing.[9]

Culture on LJ medium

From the sediment, one loopful each was inoculated onto two slopes of LJ medium using a 5 mm, 22-gauge nichrome wire loop. The date of inoculation was noted. The slopes were incubated at 37°C for a maximum period of eight weeks. They were inspected daily for growth or for contamination. In case of growth of mycobacteria, the date of appearance of the first colony was noted and the slopes were further incubated for more growth[8] [Figure 2]. The isolates obtained were confirmed as *M. tuberculosis* complex by performing niacin test, nitrate reduction test, and an immunochromatography test for the detection of MPT64 antigen.

Immunochromatography test

Two hundred µl of the buffer was added to a sterile test tube. Three colonies from the 3-4 week old culture were taken and emulsified in the buffer. The mixture was vortexed and 200 µl was taken and pipetted into the sample well and allowed for 15 minutes. A pink-colored band formation was observed at the test region along with the control region [Figure 3]. SD BIOLINE TB Antigen MPT64 Rapid test, Bangalore, India.[10]

Modified slide culture/rapid slide culture technique

The smear was done from centrifuged sediment over the lower one-third portion on one half of the longitudinally cut slide. The smear was subject to culture using human blood medium, that is, outdated, but not more than four-week old, citrated blood obtained from the blood bank was used. This blood was diluted with an equal volume of sterile deionized water to cause hemolysis. The medium was made selective by adding polymixin-B (2,00,000 units/L), carbenicillin (100 mg/L), trimethoprim (10 mg/L), and amphotericin-B (10 mg/dL) (HiMedia, Bangalore, India). The pH of the medium was adjusted to between 6.5 and 7.5. Seven milliliter of this mixture put in a sterile screw-capped McCartney bottle constituted one unit of human blood medium.

The slide was put inside the McCartney bottle containing human blood medium so that the smear part was immersed in the medium. The inoculated medium was incubated at 37°C for seven days. On the seventh day, the slide was taken out and dipped in sterile distilled water to enable washing off of the excess human blood medium. The slides was then placed in a hot air oven at 80°C for 30 minutes, stained by Zeihl Neelsen method, and examined under oil immersion for microcolonies of acid-fast bacilli[6] [Figure 1].

Statistical methods

Descriptive statistical analysis was carried out in the present study. Results on categorical measurements are presented in number (%). Significance is assessed at 5% level of significance. Diagnostic statistics like sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), accuracy, chi-square test, and the Kappa coefficient of agreement and its significance have been used.

RESULTS

A total of 200 sputum samples of patients were studied. Conventional LJ culture was taken as the gold standard test and it showed 47 (23%) positive cases. Direct smear microscopy was positive in 32 (16%) cases with a sensitivity of 68%. All the smear-positive cases were also positive by culture. Concentration using modified Petroff's method showed 39 (19.5%) positive cases and a sensitivity of 83%. Rapid slide cultures were positive in 42 (21%) cases with a sensitivity of 89%. All the above-mentioned tests showed 100% specificity.

Of 168 acid-fast bacillus — negative smears, 7, 10, and 15 cases were positive by concentration method, modified slide culture, and LJ culture, respectively.

Among the tests, direct smear microscopy and concentration method took less time for diagnosis but sensitivity was less. Rapid slide culture took lesser time (7 days) than LJ Culture (2-8 weeks) and sensitivity was good comparatively [Tables 1 and 2].

The results of both biochemical tests and immunochromatographic assay showed that 93.6% isolates belonged to *M. tuberculosis* complex and 6.4% were nontubercular mycobacteria [Table 3].

Sensitivity and specificity are the terms used to evaluate a clinical test. They are independent of the population of interest subjected to the test. The sensitivity and specificity of a quantitative test are dependent on the cutoff value above or below which the test is positive. In general, the higher the sensitivity, the lower the specificity, and vice versa. If a test is said to be highly sensitive, it means that it picks up as many positive cases as possible (both true and false positive).This is very important in diagnosing diseases like TB which are highly prevalent in an endemic country like India; hence, in our study, we looked for a test which is highly sensitive and rapid. If there are false positives, they can be subjected to further confirmatory tests for diagnosis.

DISCUSSION

TB is still a major health problem in most of the developing countries and its incidence is rising even in developed countries. This resurgence has been attributed to the HIV epidemic due to which TB has been declared as a global health emergency by the World Health Organization (WHO) in 1993.[11] The present study was undertaken to study the sensitivity and specificity of different diagnostic techniques for the detection of *M. tuberculosis* and to check the efficacy of rapid slide culture in comparison with LJ medium for the primary detection of TB. The study included 200 clinically suspected cases of pulmonary TB, excluding the patients who were on antitubercular treatment.

With respect to the above-mentioned objectives, the sensitivity of microscopy, concentration method, rapid slide culture was 68, 83, and 89%, respectively, and specificity was 100%. Among the tests which were conducted, the most sensitive method was rapid slide culture in comparison with direct microscopy and concentration methods. The following

Table 1: Correlation of direct smear findings, concentration method, and modified slide culture with LJ medium

Methods	Criteria	Total number of patients (*n*=200) (%)	LJ method		*P* value
			Positive (*n*=47) (%)	Negative (*n*=153)	
Direct smear findings	Positive	32 (16.0)	32 (68.1)	0	<0.001 highly significant
	Negative	168 (84.0)	15 (31.9)	153 (100.0%)	
Concentration method	Positive	39 (19.5)	39 (82.9)	0	<0.001 highly significant
	Negative	161 (80.5)	8 (17.1)	153 (100.0%)	
Modified slide culture	Positive	42 (21.0)	42 (89.4)	0	<0.001 highly significant
	Negative	158 (79.0)	5 (10.6)	153 (100.0%)	

Significant at 10% levels *P* value < 0.1; Significant level at 5% *P* value < 0.05; Significant level 1% *P* value is < 0.01; LJ=Lowenstein - Jensen

Table 2: Correlation of direct smear method, concentration method, and modified slide culture in relation to LJ medium for detection of *Mycobacterium tuberculosis*

Method	Sensitivity	Specificity	PPV	NPV	Accuracy	Kappa	*P* value
Direct smear method	68.09	100.00	100.00	91.07	92.50	0.765	<0.001 highly significant
Concentration method	82.98	100.00	100.00	95.03	96.00	0.882	<0.001 highly significant
Modified slide culture	89.36	100.00	100.00	96.84	97.50	0.928	<0.001 highly significant

LJ=Lowenstein — Jensen; PPV=Positive predictive value; NPV=Negative predictive value; Significant at 10% levels *P* value < 0.1; Significant level at 5% *P* value < 0.05; Significant level 1% *P* value is < 0.01

Table 3: Distribution of *Mycobacterium tuberculosis* and nontuberculous mycobacteria

Type of *Mycobacterium*	Number of patients (*n*=47)	%
M. tuberculosis	44	93.6
Nontuberculous mycobacteria	3	6.4

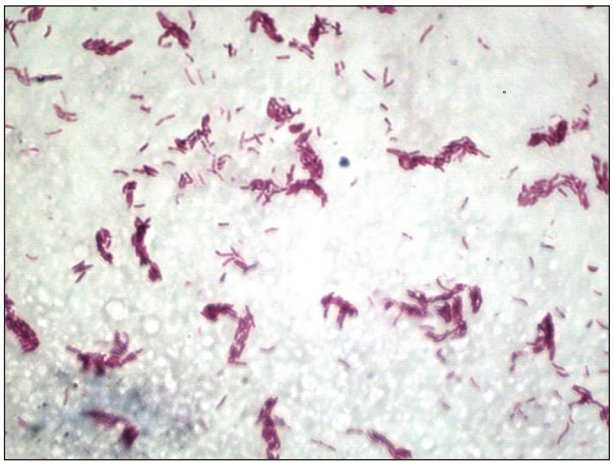

Figure 1: Ziehl Neelsen staining: Rapid slide culture showing large clumps of acid-fast bacilli; magnification 100×

Figure 2: *Mycobacterium tuberculosis:* Growth on Lowenstein - Jensen medium

Figure 3: Immunochromatography test for detection of MPT64 antigen

are the points to prove that the modified slide culture is a better method compared to other conventional methods:

a) The mycobacteria are allowed to grow on a slide without heat-fixing or killing; so, by the end of seven days, the number of mycobacteria would have increased in number compared to other (direct smear and concentration method) methods by which we would have missed the diagnosis.
b) In view of making an early diagnosis, the report can be given within a week's time compared to a culture which takes 2-8 weeks.
c) Blood is a very good medium for the growth of Mycobacteria only if the processing is done without contamination.
d) Antitubercular drug-sensitivity testing can be done by this method.
e) Microcolonies with cord formation (virulence factor) can be demonstrated.
f) Slide culture technique is recommended to be used for the management of treatment failure cases.
g) Efficacy of isolation of smear-negative cases is good.

In the present study, 32 of 200 cases (16%) were positive for acid-fast bacilli by the Zeihl Neelsen method of staining. Other studies by Lakshmi *et al.*, Jain *et al.*, Negi *et al.*, Ghatole *et al.*, and Cornfield *et al*. have shown 14.7, 18.6, 23.5, 32.7, and 33% positivity, respectively.[3,12-15]

The percentage of acid-fast bacillus positivity in the present study was comparable with that of Cornfield *et al.* and Lakshmi *et al*. The reason for the low percentage of acid-fast bacillus positivity in the present study in comparison to that of Jain *et al.* and Negi *et al.* maybe because the present study included only new cases who were clinically suspected, whereas the last two studies included, in addition, patients who were on antitubercular treatment.[11,12] The studies by Ghatole *et al*. and Jena *et al*. included radiologically proven cases and hence they show a high rate of positivity.[6,14]

Rapid slide culture was more sensitive compared to direct smear microscopy and concentration method; 42 (21%) of 200 cases were positive by modified slide culture with sensitivity and specificity of 89.4 and 100%, respectively. In a study by Jena *et al*., 105 of 336 were positive by rapid slide culture with 65.2% sensitivity. Another study by Purohit *et al*. showed 177 (93.5%) positive of 200 radiological positive cases.[5] In a study from Kozhikode, Kerala by Nair *et al*. showed 19 (23.8%) were positives out of 80 sputum samples.[16] The present study was comparable with that of Nair *et al*. and showed better sensitivity than that of Jena *et al*. The result of rapid slide culture with LJ culture showed a significant correlation in our study. An advantage of rapid slide culture is that colonies can be visualized with an ordinary bright light microscope, without the need of an

inverted microscope and results are available within seven days. Rapid slide culture proved to be a rapid, cheap, and effective method for the detection of TB. Drug-sensitivity testing can be done by using rapid slide culture technique. It is useful for the confirmation of viable TB and monitoring response to therapy.[3]

CONCLUSION

In the present study, rapid slide culture technique showed good results comparable with that of LJ culture, and this technique can be adopted in the Revised National Tuberculosis Control Program (RNTCP) as it is rapid, cheap, sensitive, and specific.

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