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Detection of *Mycobacterium tuberculosis* with conventional microscopic and culture methods

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Abstract
Background: Tuberculosis is considered as the greatest cause of death worldwide. In the developing countries including India, population density, poverty, malnutrition, and highly congested environment may create substantial risk for infection with *Mycobacterium tuberculosis.* The principal obstacle in the treatment of tuberculosis is the consumption of time and inaccurate diagnosis as well.
Materials and Methods: This retrospective study was carried out in order to compare results obtained by both the conventional AFB (Acid Fast Bacilli) microscopy and Lowenstein-Jensen (L-J) culture method for detection of *Mycobacterium* spp. in clinical samples from different categories of patients.
Results: Among one hundred and fifty samples, 83 (55.3%) AFB^+ results were found under Bright-Field (BF) microscopy, 78 (52%) AFB^+ and 91 (60.7%) AFB^+ results were observed under conventional and Light Emitting Diode (LED) fluorescence microscopy. On L-J culture media, 103 (68.7%) AFB^+ isolates were found which reveals that the culture could be a gold standard for diagnosis of TB.
Conclusion: Although AFB smear is rapid, cheap and specific test for early diagnosis of TB but its sensitivity is low and culture on LJ medium is still thought to be gold standard although takes longer time to grow and provides us with positive growth to do drug sensitivity testing.

Keywords: *Mycobacterium tuberculosis,* AFB, ZN Stain, Pulmonary TB, Lowenstein-Jensen

Introduction
Tuberculosis (abbreviated as TB for tubercle bacillus or Tuberculosis) is a common and often deadly infectious disease caused by mycobacteria in humans, mainly by *M. tuberculosis* [1]. Tuberculosis (TB) is a major air- borne infectious bacterial disease. It remains a major worldwide health problem with global mortality ranging from 1.6 to 2.2 million lives per year. The situation is further exacerbated with the increasing incidence of drug resistant TB. Multidrug resistant TB (MDR-TB) includes drug resistance to at least isoniazid (INH) and rifampicin (RIF). After HIV, tuberculosis could be considered as the leading cause of death in the world. Tuberculosis has been a major public health concern in India for long time. The country ranks 6th on the list of 22 highest burdened TB showered countries in the world. It is estimated that 3,00,000 new cases crop up each year, of which about half of them are infectious TB. It is further estimated that about 70,000 people die every year due to TB infection. Hence, each hour eight [2] persons die of the disease for which very effective treatment- Directly Observed Treatment (DOT) with free of cost, is available in India. The average TB mortality rate (45 deaths per 1,00,000 populations) in India is 45 percent higher than that in the Southeast asian region (31 deaths per 1,00,000 population) [3]. At present, diagnosis of TB due to *M. tuberculosis* (MTB) is most commonly done by using microscopy and culture methods. Most commonly Bright-Field microscope (Zeihl-Neelsen staining) and Fluorescence microscope (Auramine-O staining) are used for the detection of AFB. Most standard laboratory text books and guidelines for laboratories suggest that at least three specimens, preferably collected on three consecutive days, should be submitted to the laboratory for acid-fast bacilli (AFB) smear and culture.
In TB endemic areas, most of the cases of TB can be diagnosed correctly by simple and cheap methods which are generally available at peripheral hospital level by AFB microscopy. Although acid fast bacilli (AFB) microscopy and Lowenstein-Jensen (L-J) culture remain the cornerstone of the diagnosis of TB but these traditional bacteriological methods possess several disadvantages. They are either slow or their sensitivity is quite low,

especially with clinical samples that contain small number of organisms. This can adversely affect the treatment by either delaying it or by causing inappropriate empiric therapy for TB to subjects without mycobacterial infections or with atypical mycobacteria [2]. Therefore, an urgent requirement of promoting highly sensitive LED fluorescence microscopy led us to design the present study.

Materials and methods

Study site and study population. The study was carried out at Department of pulmonary medicine, IMS and SUM hospital, from January 2014 to December 2016. Patients of all ages and both sexes who were treated with anti-tubercular drugs but did not respond and patients who did not receive any anti-tubercular drugs were enrolled in the study.

Procedure of sputum collection. One spot of morning sputum sample was collected in a container. On the initial hospital visit, the patient was provided a clean, dry, sterile wide-neck, leak-proof container and requested him or her to cough deeply to produce a sputum specimen.

Microscopic observation. Two direct smear were prepared for staining by Ziehl-Neelsen (Z-N) and Fluorescence (FM) techniques respectively and were examined under microscope following standard operating procedure.

Smear preparation and fixation. For Ziehl-Neelsen staining and auramine-O staining, smears were prepared from yellow purulent portion of the sputum using a sterile bamboo stick. A good smear was spread evenly, 3 cm by 2 cm in size in the middle part of the slide which was neither too thick nor too thin. The sputum was left for 15-30 minutes for air drying. The smear was fixed by placing the slide over the hot plate at 85 ^{o}C for about 3-5 minutes.

Method of conventional bacteriological culture. Lowenstein-Jensen (L-Z) medium is the most widely used matrix for tuberculosis culture. L-J medium containing glycerol favors the growth of *M. tuberculosis*. After decontaminating and concentrating procedure of sputum samples, three or four drops of deposit were inoculated on two slopes of Lowenstein-Jensen media. The media were incubated at 37 ^{o}C after inoculation. They were examined within 3-5 days after inoculation for early recognition of rapidly growing *Mycobacterium* and of contaminated cultures, followed by examination once a week for at least 45 days. Culture was reported as positive as soon as colonies of characteristic morphology constituted of acid-fast bacilli were recognized. The report of culture was prepared according to the number of colonies.

The final species identification of *M. tuberculosis* was done based on the characteristics such as slow growth, colony morphology, and the typical biochemical features. The identities of the isolates were made by growth rate, colony morphology, *p*-nitrobenzoic acid (PNB) susceptibility, catalase and nitrate reduction tests.

Results

Among one hundred and fifty patients, in L-J culture method a total of AFB^{+} (Fig 1) results were detected in 103 (43.3%) cases and AFB^{-} results in 47 (31.3%) cases. In BF microscopy, 83 (55.3%) cases were detected as AFB^{+} results and 67 (44.7%) cases as AFB^{-}. In conventional and LED fluorescence microscopy, AFB^{+} results were detected in 78 (52%) cases and 91 (60.7%) cases and negative in 72 (48%) and 59 (39.3%) cases (Table 1).

A total of four cases were AFB^{+} in BF microscopy, but were negative in L-J culture method among one hundred and fifty patients. In twenty cases, AFB^{+} isolates were observed in L-J culture method which was negative in BF microscopy. AFB^{+} results were detected in 84 cases for both of the methods and in forty two cases it was negative. These results also indicated that the sensitivity of BF microscopy was 95.6% and specificity was 91.3% compared to the L-J culture method. All the data were statistically analyzed to predict the sensitivity, specificity, accuracy, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of BF microscopy against culture method which were 95.6%, 91.3%, 100%, 80.7% and 67.7%, respectively (Table 2).

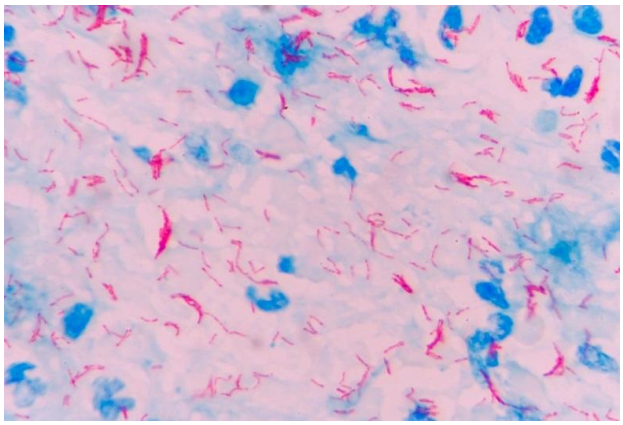

Fig 1: M tuberculosis on ZN stain

Table 1: Results of L-J culture, BF microscopy, conventional fluorescence microscopy and LED fluorescence microscopy

Methods specific for *M. tuberculosis*	No of samples		P value
	Positive (%)	Negative (%)	
L-J culture	103 (43.3)	47 (31.3)	0.000S
BF microscopy	83 (55.3)	67 (44.7)	0.083NS

Table 2: Comparison of results of BF microscopy with culture method to detect *M. tuberculosis*

BF microscopy	Culture results		Total
	Positive	Negative	
Positive	84	4	88
Negative	20	42	62
Total	104	46	150

Among one hundred and fifty patients, six cases gave AFB^{+} results in conventional fluorescence microscopy, but negative in L-J culture method. In twenty five cases, AFB^{+} isolates were observed in L-J culture method which was negative in the conventional fluorescence microscopy. AFB^{+} results were detected in seventy nine cases for both of the methods and in forty cases it was found to be negative. These results also indicated that the sensitivity of conventional fluorescence microscopy was 92.9% and the specificity was 86.9% compared to those from the L-J culture method. All the was statistically analyzed to predict the sensitivity, specificity, accuracy, Positive Predictive

Value (PPV) and Negative Predictive Value (NPV) of conventional fluorescence microscopy against culture method which were 92.9%, 86.9%, 100%, 92.9% and 61. 5%, respectively.

Discussion

Among communicable diseases, tuberculosis is the second leading cause of death worldwide, killing nearly two million people each year. Most cases are in under developed countries of the world [5]. Another significance of the current work is the statistical analysis on the sensitivity and specificity pattern of AFB microscopic methods. The sensitivity and specificity of BF microscopy was 95.6% and 91.3%. The sensitivity was 92.9% and specificity was 86.9% in conventional fluorescence microscopy. In LED fluorescence microscopy, the sensitivity and specificity was 97.9% and 95.2%, respectively. Thus, LED fluorescence microscopy had higher sensitivity and specificity than others. In a similar study, Githui *et al*. (1999) compared the reliability of fluorescence microscopy (FM) and Ziehl-Neelsen (Z-N) staining method for examination of direct smear in the diagnosis of pulmonary tuberculosis. Culture results were used as the gold standard for assessment [6]. Specificity was 97% and 96% for FM and Z-N methods, respectively. The sensitivity of the FM method was 80% than that of the Z-N method 65%. So, these studies revealed with the present consistancy study. Fluorescent microscopy offers well-described benefits, compared with conventional light and fluorescence microscopy, for the evaluation of sputum smear samples for tuberculosis and reduces unnecessary labor.

Thus, this study indicates that in the diagnosis of TB, LED fluorescence microscopy had greater sensitivity and specificity than other microscopic methods. In particular, in case of a single specimen, the diagnostic value of culture was quite significant. It is, therefore, possible to conclude that both microscopy and culture can be used for the diagnosis of TB. If only one or two specimens are available, culture method is preferable. The Z-N method has commonly been used around the world, particularly in developing countries, because of its simplicity and low cost [7-11].

The sensitivity of AFB microscopy (71%) for pulmonary specimens in this study is almost similar to that reported by other studies [11, 12]. However one study also reported the high sensitivity of AFB smear microscopy up to 75%13. Another study conducted in the same center reported sensitivity of 66.23% for pulmonary specimens8 which is little low as compared to the present study. This could be due to the fact that most of the specimens received in our study came from patients suspected clinically and radiologically to have pulmonary tuberculosis. Three sputum smears for acid-fast bacilli are recommended for proper diagnosis in pulmonary suspects of TB15. However, WHO has proposed two smears for the diagnosis of TB in countries having functional external quality assurance8. Culture using LJ medium has been the gold standard for the diagnosis of tuberculosis for many years in the developing countries [7]. An overall AFB culture positivity in the present study was 15.47% and is little higher than the study that revealed the culture positivity of 12.3% 14.While others have reported a culture positivity of 48.9% and 47.1% respectively 12, 15. Culture positivity in the present study is significantly high as compared to AFB smear microscopy as about 5000 to 10000 AFB/ml of specimen is needed to yield positive result by AFB smear microscopy while the advantage of culture on LJ medium is that it has the sensitivity of 80-85%, very specific and being able to detect as few as 10 bacteria per milliliter of specimen [16, 17].

Conclusions

The emergence of MDR-TB poses a significant threat to TB control activities throughout the world. The complexity of MDR-TB management makes it essential to create new skills to design, plan, implement, and monitor interventions for the management of MDR-TB. More surveillance and immediate therapeutic interventions should be performed in order to combat the threat of MDR-TB to the general population. At present the diagnosis of tuberculosis in India is based on AFB (acid fast bacilli) microscopy using Z-N staining. Though it is cheaper and faster, its sensitivity and specificity is less. Therefore, it is necessary to initiate the use of LED fluorescence microscopy for more accurate and rapid detection. The conventional cultural diagnosis is considered as gold standard for diagnosis of TB but is inappropriate for immediate treatment of tuberculosis. These conditions reflect the need of new diagnostic procedure especially molecular methods for detection of tuberculosis in our country as it requires only two days where conventional culture diagnosis takes more than four weeks. Proper selection and use of anti-tubercular drugs for treatment of patients is also necessary to reduce the incidence of MDR-TB.

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