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Research Article

Light Emitting Diode (LED) based Fluorescent Microscopy versus Bright Field Microscopy for the Diagnosis of Tuberculosis from Extrapulmonary and Non-sputum Pulmonary Samples

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Abstract Microscopy is the mainstay in the diagnosis of tuberculosis (TB) in resource poor settings. Usefulness of Auramine-O staining and LED (AO-LED) microscopy for the diagnosis of tuberculosis from extrapulmonary and non-sputum pulmonary samples was evaluated. A prospective study was performed on 75 extrapulmonary samples like CSF, Urine, Pleural fluid and non-sputum pulmonary samples like BAL. Smears were stained with auramine-O and Ziehl-Neelsen (ZN) stains and viewed under LED microscopy and bright field(BF) microscopy respectively. Solid culture (Lowenstein-Jensen medium) was used as a reference standard. Out of the 75 specimen collected, 24% of them were CSF followed by BAL specimen (23%) and pleural fluid (13%). Three (4%) were positive by AO-LED microscopy whereas only one (1%) was positive by ZN-BF microscopy. The percentage of TB positivity among the three different methods used, revealed a high yield by culture (8%), followed by AO-LED (4%) and ZN-BF (1.3%) methods. AO-LED microscopy had a better sensitivity of 50% Vs 16.6% in comparison to ZN-BF microscopy. Both AO-LED and ZN-BF microscopy methods had a specificity and positive predictive value of 100%. Both the techniques had a negative predictive value of 95.8% and 93.2% respectively. We found a higher smear positivity rate with AO-LED in comparison with ZN-BF microscopy. The AO-LED microscopy was found to be more sensitive than the ZN-BF (50% Vs 16.6%). We report that it would be prudent to use AO-LED microscopy for non-sputum samples like BAL replacing the current practice of ZN-BF microscopy for the diagnosis of smear negative pulmonary tuberculosis.

Keywords Auramine-O, Ziehl-Neelsen, BAL, Tuberculosis, LED microscope

1. Introduction

Tuberculosis is a major cause of morbidity and mortality in the developing world. It is the leading cause of death amounting to 1.3million deaths worldwide, due to a single infectious agent ranking above HIV/AIDS. Tuberculosis (TB) patients in India accounts for nearly 1/4th of the total global burden of TB. The total estimated incidence of TB in India in the year 2016 accounted to nearly 2.28 million cases out of a global incidence of 10.4 million [1]. All these signify the importance of early diagnosis and treatment of tuberculosis as most of these deaths could have been prevented. In the advent of the 21st century, newer and rapid molecular diagnostic methods like CB-NAAT (cartridge based nucleic acid amplification) and LPA (Line probe assay) methods took the lead in the diagnosis of tuberculosis. However, in developing countries like India the use of these expensive methods pose an economic burden, with the national budget for TB prevention and care doubling from 280 million USD in 2016 to 525 million USD in 2017 [1]. This financial limitation would render expensive molecular methods unavailable at the point of care centers, and leaves only the economically feasible and time tested microscopy and culture as the solution.

Though culture is described as the gold standard, it requires long incubation periods like 1-3 weeks and 3-8 weeks for liquid and solid cultures respectively [2]. Apart from the long turnaround time for obtaining results, culture is performed only at specialized laboratories particularly the reference laboratories, where specialized equipment and trained staff are available [3]. On the contrary, microscopy is rapid, less labour intensive, does not require specialized equipment and are very much available and suitable for

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primary health centers [4]. In a resource poor setting, the diagnosis of pulmonary and extrapulmonary tuberculosis relies on identification of acid fast bacilli by the Ziehl-Neelsen (ZN) staining and bight field microscopy. The ZN microscopy is highly specific but lacks sensitivity (20-80%) [4].

Of late the Light-emitting diode (LED) based fluorescent microscope using the auramine-O staining has been found to have an increased sensitivity by 10% [5]. The World Health Organization (WHO) and the Revised National Tuberculosis Control Program (RNTCP) have recommended the use of Auramine-O staining and Light-emitting diode (LED) microscopy for the diagnosis of pulmonary tuberculosis [6, 7]. Previous studies reporting the usefulness Auramine-O LED fluorescence (AO-LED) microscopy in comparison to ZN bright field microscopy (ZN-BF) have been done exclusively for pulmonary TB suspects and on sputum samples [8-10].

Diagnosis of TB from extrapulmonary sites and non-sputum pulmonary specimen like bronchoalveolar lavage (BAL) and gastric aspirates still relies on ZN microscopy. There is paucity in data on evaluating Auramine-O LED microscopy for the diagnosis of extrapulmonary tuberculosis. Hence, we in this study intend to evaluate the usefulness of AO-LED microscopy for the diagnosis of tuberculosis from extrapulmonary and non-sputum pulmonary samples.

2. Materials & Methods

2.1. Setting

This prospective study was performed during the period of August 2016 to November 2016 at the designated microcopy center (DMC) of the department of Microbiology, Pondicherry Institute of Medical Sciences, a tertiary care center located in the Union territory of Pondicherry, India. The study was approved by the Institutional research and ethics committee.

2.2. Specimen Collection

Seventy five extrapulmonary samples like cerebro-spinal fluid (CSF), peritoneal /pleural fluid, urine, tissue, ascitic fluid, bone marrow from suspected TB patients that were received in the department of microbiology were included in the study. Non-sputum pulmonary samples like gastric aspirates and Bronchoalveolar lavage (BAL) was also included in the study.

2.3. Preparation of Stains

Ziehl-Neelsen stain and the Auramine-O stain reagents were prepared according to the standard recommendations as per the RNTCP guidelines as described earlier [11, 12]. The reagents were prepared and stored in dark bottle and stored at room temperature till further use.

2.4. Preparation of Smear and Staining

All specimens were centrifuged at 2000 RPM for 10 minutes. With an inoculating loop the deposit were smeared over 2 grease-free clean glass slides. The slides were numbered appropriately and the details were blinded to the microscopist.

The Smears were heat fixed and stained according to the standard RNTCP guidelines as described earlier [11, 12]. Staining was done in duplicates using the Ziehl-Neelsen staining (Conc.carbol fuchsin, 25% sulphuric acid and 0.1% methylene blue) for bright field microscopy and Auramine-O staining (Auramine-phenol, 1% acid-alcohol and 0.1% potassium permanganate) for LED microscopy.

2.5. Microscopy

The Ziehl-Neelson stained smears were then viewed under Olympus-*CH20i* bright field microscope (Magnification-1000X) and Auramine-O smears under Carl Zeiss-*Primo Star iLED* microscope (magnification-400X).

2.6. Culture

After decontamination and concentration by modified Petroff's method using 4% NaOH, the specimen was inoculated onto two sets of Lowenstein-Jensen (LJ) medium and one LJ slope with Para-nitrobenzoic acid (500µg/ml). The inoculated media were incubated at 37℃ and observed weekly for visible growth of colonies for upto 8 weeks. When macroscopic growth was observed, colonies were picked up and confirmed by ZN staining.

2.7. Statistical Analysis

All the parameters were analysed descriptively using statistical methods. The data obtained based on count were expressed as either numbers or percentages (%). The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of Zeihl-Neelsen-Bright field (ZN-BF) microscopy and Auramine-O-LED (AO-LED) microscopy were determined, comparing it with culture as the gold standard.

3. Results

A total of 75 extrapulmonary and non-sputum pulmonary samples were collected from tuberculosis suspects during the study period. Majority of the samples 44(58.6%) were from males followed by 31(41.3%) from females. The age of tuberculosis suspects ranged from 4 months to 77 years. Majority of the patients 37(49.3%) were from the middle aged (18-59) group followed by elderly 30(40%) and 8(10.6%) in the pediatric age group.

Out of the 75 specimen collected, 24% of them were CSF followed by BAL specimen which was 23%, followed by pleural fluid (13%). (Figure 1).

Out of the 75 specimen that were stained, 3(4%) were

positive by AO-LED microscopy whereas only one (1.3%) was positive by ZN-BF microscopy. The ZN positive smear was also positive by AO-LED microscopy, but 2 of the smears that were positive by AO-LED microscopy were ZN-BF negative (Table 1) (Figure 2).

Table 1. Comparison of AO-LED with ZN-BF microscopy

	AO Positive	AO negative	Total
ZN Positive	1	0	1(1.3%)
ZN Negative	2	72	74
Total	3(4%)	72	75

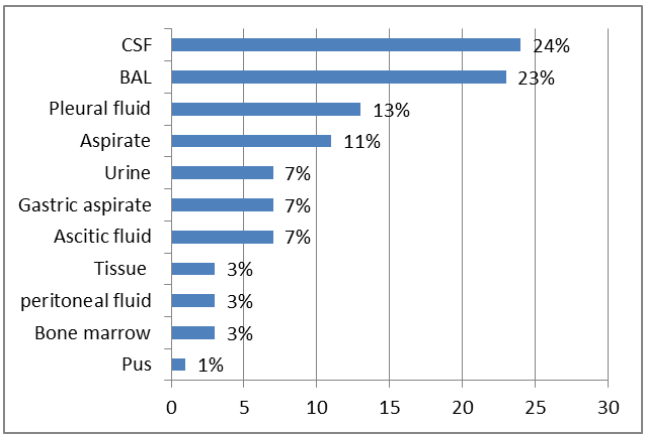

Figure 1. Distribution of different specimen received from tuberculosis suspects

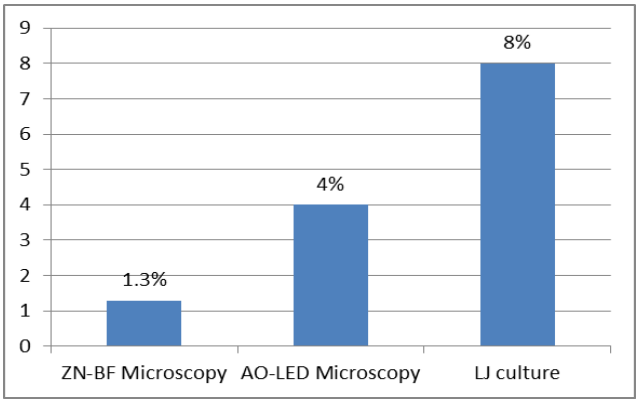

Figure 2. Percentage of positivity of the three diagnostic methods performed among TB suspects

Table 2. Performance of AO-LED and ZN-BF microscopy in comparison with the gold standard – culture

	AO-LED microscopy		ZN-BF microscopy	
	Positive	**Negative**	**Positive**	**Negative**
Positive LJ culture	3	3	1	5
Negative LJ culture	0	69	0	69
Total	3	72	1	74

Six samples (8%) grew on LJ medium out of which 3(100%) samples were positive by AO-LED microscopy. However, 3(50%) other samples that were culture positive were negative with AO-LED microscopy and 5(83%) of the culture positive were negative by ZN-BF microscopy (Table 2).

All three samples that were positive by AO-LED microscopy were BAL samples. The sputum microscopy results of these patients were reviewed. One patient was found to be sputum smear-negative by AO-LED microscopy and other 2 patients were found to be positive.

When compared with solid culture as a reference standard, the AO-LED microscopy had a better sensitivity of 50% in comparison to ZN-BF microscopy which was only 16.6%. Both AO-LED and ZN-BF microscopy methods had a specificity and positive predictive value of 100%. Both the techniques had a negative predictive value of 95.8% and 93.2% respectively (Table 3).

Table 3. Sensitivity, specificity, PPV and NPV of the AO-LED and ZN-BF microscopic methods

	AO-LED microscopy	ZN-BF microscopy
Sensitivity	50%	16.6%
Specificity	100%	100%
Positive predictive value (PPV)	100%	100%
Negative predictive value (NPV)	95.8%	93.2%

4. Discussion

Microscopy is the mainstay in the diagnosis of pulmonary and extrapulmonary tuberculosis in the resource poor setting. Auramine-O staining and LED microscopy has been extensively evaluated and is currently in use for sputum microscopy. However, extrapulmonary samples and non-sputum samples like BAL are still being stained by the conventional ZN stain and viewed by the bright field microscopy which has a very low sensitivity. The present study evaluated the role of AO-LED microscopy in the diagnosis of TB from extrapulmonary and non-sputum samples like BAL.

A vast majority of TB suspects in our study were males 44(58%), which is in consensus with a study done by Austin et al, which could be attributed to the better health seeking behaviour among male population [13]. Most of them (49%) also belonged to the middle age group. TB meningitis was the most suspected clinical condition in our study, hence CSF samples constituted the highest number of samples followed by BAL.

We found a higher smear positivity rate with AO-LED in comparison with ZN-BF microscopy, in concurrence with previous studies [13, 14]. This can be explained by the fact that fluorescent colour offers a better contrast against a black background in AO-LED than pink against a blue background as in ZN-BF (Figure 3). Moreover, AO-LED microscopy uses a magnification of 400X covering a larger field of the slide. ZN-BF uses a bigger magnification of 1000X and covers a smaller field thereby increasing the probability of missing the bacilli.

The AO-LED microscopy was found to be more sensitive than the ZN-BF (50% Vs 16.6%), and had a higher negative predictive value (95.8% vs 93.2%), however had similar specificity (100%). Previous studies have demonstrated similar findings, which is possibly due to a stronger affinity of auramine to mycolic acid of the tubercle bacilli than carbol fuchsin [14-16]. Though previous studies have evaluated AO-LED on sputum samples, the findings from our study on BAL (non-sputum pulmonary samples) is in agreement with their findings.

The percentage of TB positivity among the three different methods used, revealed a high yield by culture, followed by AO-LED and ZN-BF methods (Figure 2). Though the microscopic methods are conventionally labeled as more sensitive than specific, the load of the bacilli has to be atleast 5000-10,000/ml to be picked up by microscopy [17]. On the contrary, just 10-100 bacilli are required for culture positivity [18]. This explains the high positivity rate by culture than microscopy in our study. Moreover, in our study AO-LED did not pick up acid fast bacilli from extrapulmonary specimen where the bacillary load is usually very low.

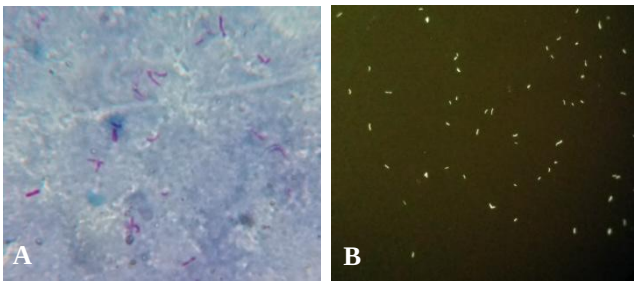

Figure 3. Images showing microscopic picture of Acid fast bacilli, (A)-Ziehl-Neelson stained smear viewed under bright field microscopy (1000 X magnification) and (B) Auramine-O stained smear under LED microscopy (400 X magnification)

In our institution BAL samples are collected as a routine from sputum smear negative patients on whom there is a high suspicion of tuberculosis. Hence, sputum microscopy results as well as the BAL microscopy results were available for all the pulmonary TB suspects that were included in the study. Among three patients whose BAL were positive by AO-LED microscopy and culture, one of the patient was found to be sputum smear negative. His BAL sample showed negative results by ZN-BF microscopy too, which means that had he been subjected to sputum and BAL microcopy by ZN method alone as per the guidelines, the diagnosis of tuberculosis by direct evidence could have been missed. Missing out on diagnosis of tuberculosis can lead to fatal life threatening complications. In concordance to our study, Bodal VK et al have concluded in their study that over 50% of smear-negative hidden cases of TB were diagnosed by bronchoscopic specimen stained and viewed by AO-LED microscopic method [19]. The sample size of our study was small which is a limitation of this study, however in a country like India where the annual incidence of TB is accounted in millions, many TB cases might go undiagnosed. This will significantly increase the incidence and prevalence of TB and hinder the eradication of tuberculosis.

5. Conclusions

This study evidently highlights the importance and advantage of the AO-LED microscopy for diagnosis of pulmonary tuberculosis from non-sputum pulmonary samples like BAL. The increased and superior sensitivity of AO-LED over ZN-BF (50% Vs 16.6%) and better negative predictive value (95.8% vs 93.2%) proves the above statement. Our study also showed the improved diagnostic value of AO-LED microscopy in detecting tuberculosis in sputum smear negative patients. The current practice of ZN-BF microscopy for Staining BAL samples had missed the diagnosis of TB in one of our sputum smear negative patients. However, AO-LED microscopy picked up the bacilli in the same patient.

The fluorochrome stain (Auramine-O) and the LED microscope is far more superior and efficient in detecting acid fast bacilli from BAL samples due to the better affinity of the stain to the bacilli and moreover, larger area can be viewed in a single field. This may be further accentuated by studies using larger sample size.

In conclusion, we report that it would be prudent to use AO-LED microscopy for non-sputum samples like BAL replacing the current practice of ZN-BF microscopy for the diagnosis of smear negative pulmonary tuberculosis.

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