Comparison of Light Emitting Diode (LED) Fluorescent Microscopy with Ziehl-Neelsen Microscopy on Sputum Specimens for Diagnosis of Pulmonary Tuberculosis Keeping Culture as 'Gold Standard'

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ABSTRACT

Objective: To compare LED fluorescence microscopy and Ziehl-Neelsen staining in terms of their diagnostic performance in diagnosing pulmonary tuberculosis, taking sputum specimens from patients suspected of pulmonary tuberculosis.
Study Design: Prospective longitudinal study.
Place and Duration of Study: Microbiology Department, Armed Forces Institute of Pathology Rawalpindi, from Jan 2019 to Dec 2019.
Methodology: Sputum samples from patients with clinical suspicion of pulmonary tuberculosis were stained using Ziehl-Neelsen (ZN) stain, fluorescent stain with Auramine O staining (AO) stain and Mycobacterial culture on Mycobacterial Growth Indicator Tube (MGIT 960), to detect Mycobacterium tuberculosis. WHO guidelines were followed to grade positive smears.
Results: Among 206 patients with suspicion of tuberculosis, 143 (69%) were male, and 63 (30%) were female patients. The mean age of the patients was 53.67 ± 14.73 years. Out of 206 sputum samples, 64 were negative by all three techniques used. 142 (68%) specimens detected Mycobacterium tuber-culosison MGIT960. Within 142 culture-positive samples, only 40 samples were positive on Ziehl-Neelsen microscopy, whereas 97 samples were detected positive by LED fluorescent microscopy. In culture-negative samples, three were missed on Ziehl-Neelsen staining, which was positive with Fluorescent microscopy. Sensitivity and specificity for Ziehl-Neelsen smear microscopy were 26.7% and 96.8%, respectively. Sensitivity and specificity for Fluorescent smear microscopy were 64.8% and 92.2%, respectively.
Conclusion: We concluded that the efficacy of LED fluorescence microscopy has proven to have many potential advantages over conventional Ziehl-Neelsen microscopy.

Keywords: Auramine-o fluorescent stain, Light-emitting diode (LED) fluorescent microscopy, Mycobacterium tuberculosis, Ziehl-neelsen stain.

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INTRODUCTION

Tuberculosis (TB) is a global public health problem caused by the bacilli, a member of the Mycobacterium tuberculosis complex i.e., Mycobacterium tuberculosis.[1] Tuberculosis (TB) is one of the oldest diseases afflicting human beings. About 9 million cases are reported annually world-wide.[2] WHO report in 2015 reported 10.4 million incident cases of TB that took away the life of 1.4 million people.[3] Pakistan ranks sixth among the countries which account for almost 60% of the total TB burden globally.[4] More than 95% of deaths due to tuberculosis occur in low-to middle-income countries.[5] Despite the advent of novel diagnostic advances for tuberculosis (TB), conventional microscopy remains the most practical and rapid test offered in resource-limited setups. Mycobacterial culture is the 'gold standard for TB diagnosis. However, the turnaround time for culture is 3–6 weeks.[6] Therefore, considerable efforts and resources are being invested in developing novel diagnostics and improving existing ones to make a prompt diagnosis. Although currently, Ziehl-Neelsen staining (ZN) is the most commonly used method to detect AFB in suspected sputum specimens, it is less sensitive and has low specificity. The introduction of fluorescent microscopy has become more helpful in detecting AFB as it is more sensitive than ZN staining. It is less cumbersome, has simple diagnostic criteria, and requires a single specimen to decide.[7] Those cases that were missed before by smear microscopy due to fewer bacilli can easily be picked by LED fluorescent (LED-FM) microscopy. This helps avoid delay in diagnosis and subsequent initiation of treatment, thus preventing further spread

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of the disease.[8] The study intended was to determine the diag-nostic accuracy of LED-FM and conventional ZN stain-ing taking Mycobacterial tuberculosis culture as the "Gold Standard" for resource-poor settings.

METHODOLOGY

This prospective longitudinal study was carried out from January to December 2019 at the Microbiology Department of Armed Forces Institute of Pathology (AFIP), Rawalpindi. The study was approved by the Institutional Ethical Review committee certificate numbe FC-MIC13-4/READ-IRB/17/327. Additionally, informed consent was taken from each patient. Non-probability convenient sampling technique was used for sample collection.

Inclusion Criteria: Sputum samples from the patients of either gender and age group, with clinical suspicion of pulmonary tuberculosis were included in the study.

Exclusion Criteria: Previously diagnosed PTB patients, repeat sputum specimens of the same patient, patients with a history of lung malignancies or fungal infections and inappropriately collected samples (i.e., the salivary sample), and other pulmonary samples except for sputum and extrapulmonary samples were excluded from the study.

Clinical suspicion of pulmonary TB was based on the patients' history, signs, symptoms, and radiographic assessment. Spot sputum specimens were taken from all the patients. After taking the fitness of the collected sputum sample, all the specimens were processed and treated with standard N-acetyl-L-cysteine (NALC) and 2% sodium hydroxide (NaOH) for decontamination and digestion, which was then centrifuged (3000 × g) for about 15-20 minutes to make a deposit, which was after that utilized for staining and culture simultaneously.

For ZN staining smear was prepared on a glass slide by taking 1-2 drops from the resuspended specimen deposit, then dried by putting it into a hot air oven (at 56°C) for about 5 minutes. After that, standard ZN staining steps were undertaken. According to WHO guidelines, the result was reported and graded as negative, scanty, 1+, 2+ or 3+ according to several tubercle bacilli seen under the microscope.

The smear was flooded with Auramine-O stain for 15-20 minutes for fluorescent microscopy. After rinsing the smear with water, acid alcohol was used to decolourize it for 30 seconds. Finally, the smear was counterstained using methylene blue for one minute. AFBs were examined using an LED fluorescent microscope with 400x magnifying power. The tubercle bacilli were identified easily, thus causing less eye strain. The sample was categorized as negative, scanty, +1, 2+, 3+ based on several bacilli present in 1-length (40-50 fields) as per WHO guidelines.[9,10]

For culture, all the specimens were subjected to the digestion and decontamination process by the standard peter off method. The samples were inoculated and incubated into a fully automated MGIT 960 TB system, positive control (using ATCC 25177 Mycobacterium tuberculosis strain), negative control, i-e, and un-inoculated MGIT tube. After a signal flashed positive, the tube was taken out of the MGIT 960 system. Afterwards, ZN staining was done to confirm the presence or absence of AFB.

The quality of the reagents was checked by staining a known positive smear by following standard operational procedure. Statistical Package for Social Sciences (SPSS) ver-sion 20.0 was used for the data analysis. Diagnostic parameters were calculated using a 2 x 2 table. Sensitivity, specificity, positive predictive value, negative predictive value and diagnostic accuracy were deter-mined by using the standard formulae.

RESULTS

Total 206 sputum specimens were included and examined for AFB. The mean age of the patients was calculated as 53.67 ± 14.73 years. There were 143 (69%) male patients and 63 (30%) female patients. One patient presented with a relapse of tuberculosis five years after getting completely cured and was on anti tuberculous treatment (ATT) again, while 205 patients were new cases. Among 206 sputum samples, 64 (31%) samples were tested negative by all three modalities. However, 142 (68%) samples were detected AFB positive on MGIT960. Out of 142 samples that were culture positive, 40 (28%) were smear-positive by ZN microscopy, and 97 (68%) specimens were AFB positive by LED fluorescent microscopy as shown in the Figure.

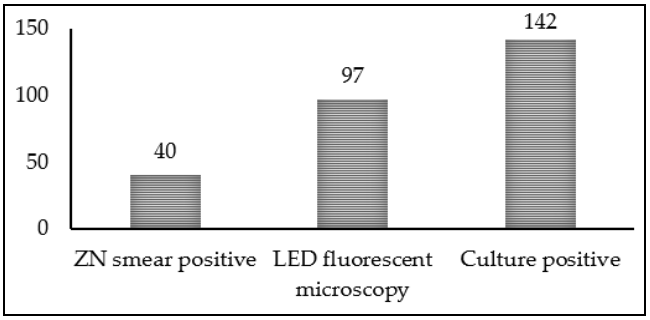

Figure: Number of acid fast bacilli (AFB) detected by three modalities (n=206).

Out of culture-negative samples, five (05) samples were false positive with Fluorescent staining and subsequent microscopy and two with ZN staining. Therefore, overall positivity was increased with fluorescent smear microscopy that, on further testing, when confirmed, were found to be Mycobacterium other than tubercle bacilli(MOTT). The difference in case detection was statistically significant (p<0.001). The sensitivity, specificity, PPV, and NPV of ZN microscopy and LED- FM were shown in Table.

Table: Diagnostic accuracy of Ziehl-Neelsen staining and LED fluorescence microscopy (n=206).

Microscopy		Culture					
		Positive (%) 142 (68)	Negative (%) 64 (31)	Sensitivity (%)	Specificity (%)	Positive Predictive Value (%)	Negative Predictive Value (%)
Ziehl-Neelsen Microscopy (Conventional)	Positive (%) 40 (19)	38 (18)	02 (1)	26.7	96.8	95	37
	Negative (%) 166 (81)	104 (50)	62 (30)				
Fluorescent Microscopy	Positive (%) 97 (47)	92 (45)	5 (2)	64.8	92.2	94.8	54
	Negative (%) 109 (53)	50 (24)	59 (29)				

DISCUSSION

Pulmonary tuber culosis (PTB) is one of the oldest diseases that still affects humankind badly. Globally the total number of cases of pulmonary tuberculosis is continuously on the rise despite major control initiatives done internationally so far.[11] Although there is a variety of novel diagnostic techniques and modalities for the diagnosis of TB used in many resourceful settings, smear microscopy remains one of the most widely used inexpensive points of care diagnostic tests available for underdeveloped countries.[9,10] Early diagnosis and prompt initiation of treatment is significant for therapeutic reasons and to control the spread of its epidemic. Although conventional direct smear microscopy has very low sensitivity for the diagnosis of tuberculosis, it remains foremost rapid, inexpensive, highly specific, and capable of identifying the most infectious cases of TB.[4,5,10] The main limitation of the method is its low sensitivity. A variety of factors may influence the overall sensitivity of ZN microscopy, such as smear preparation, technical work experience of the viewer and amount of AFB present in the sample.[11] There was a high percentage of tuberculosis positivity from early morning sputum samples compared to the first spot and second spot sputum samples when using ZN staining technique. Recently, Light-emitting diode(LED) fluorescence microscopy is an alternative technique with increased sensitivity compared to ZN microscopy.[12,13] LED fluorescence microscopy (LED-FM) has several potential diagnostic advantages over conventional Ziehl-Nelson microscopy. LED fluorescence microscopy is cost-effective, and smears can be examined much less time than needed for ZN smears, thus causing less eye strain to diagnose TB with more comprehension. LEDs ignite Auramine; therefore, UV light is not produced. It does not need a darkroom in contrast to conventional fluorescent microscopy. Fluorescent acid-fast bacilli are viewed at lower magnification. However, usage of LED-FM requires appropriate technical education and strict quality control.[13] World Health Organization recommends Light-emitting diode fluorescence microscopy (LED-FM) to replace conventional Ziehl-Neelsen microscopy for the diagnosis of pulmonary tuberculosis.[14]

In our study, the sensitivity of ZN staining was 26.7% whereas the sensitivity of fluorescent microscopy was 64.8%. A similar study comparing the sensitivities and specificities of both microscopies was conducted in India by Hooja *et al.* According to this study, the sensitivity of ZN stain was 55.5%. In comparison, that of Fluorescent microscopy was 71.85%. The specificity for ZN and fluorescent staining was 99.2%.[15] Another study by Laifangbam *et al,* done in India concluded that the efficacy of fluorescent microscopy 71% is far better than ZN 44% microscopy in detecting acid-fast bacilli in the same suspected sputum sample. The results of the two above mentioned studies concluded that fluorescent microscopy is superior to ZN microscopy, just like our study, except that both studies have higher values of sensitivity and specificities for both techniques than the values in our study. However, the positive predictive values for fluorescent microscopy were almost comparable in both studies.[16] The results of sensitivity and specificity of fluorescent stain according to a study conducted by Cattarmanchi *et al,* were 72% and 81%, respectively. The results of our study are not per the study mentioned earlier as in our study LED-FM has a lower value for the sensitivity i-e., 64.8% but a relatively higher value i.e. 92.2% for the specificity, respectively.[17] Our results for conventional ZN microscopy are comparable with a similar study conducted by Agarwal *et al.*[18] However, we reported a higher sensitivity value. A

study in Bangladesh reported that LED–FM has higher sensitivity and specificity 95.38% and 94.11%, respectively, than ZN microscopy 56.06% and 97.61%; however, both have higher values compared to our study.[19] Khalil *et al,* reported similar results for ZN microscopy. However, this study had a significantly lower negative predictive value of ZN microscopy.[20] The results of our study are not in accordance with another study done in a district hospital in Ghana that concluded sensitivity of ZN smear microscopy and fluorochrome stain was 54.8% and 84.5% respectively, specificity of both were 100% according to this study which is higher than our values reported.[21] We concluded that LED fluorescent microscopy showed superior diagnostic performance than conventional ZN smear microscopy. Considerable effort and resources have been implemented to develop novel diagnostics parameters and to improve the existing ones.[22,23]

Conflict of Interest: None.

Authors' Contribution

FTZ: Data collection, analysis, MZS: Data analysis, Statistics, IAM:, SQZ: Data collection, analysis, FS:, AH: Data collection.

CONCLUSION

Timely diagnosis and initiation of prompt treatment of TB remain pivotal to breaking its transmission chain. LED Fluorescence microscopy has shown much superior performance and higher diagnostic yield with a high positivity rate than Ziehl-Neelsen microscopy. Therefore, for a sparse resource country like Pakistan, especially where tuberculosis is endemic, fluorescent microscopy is assumed to be more advantageous and helpful in making a timely diagnosis of highly transmittable disease.

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