Different Methods of Staining to optimize Smear Microscopy for Diagnosis of Pulmonary and Extra Pulmonary Tuberculosis

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Abstract

Introduction: Tuberculosis continues to be a major public health problem especially in low income and high incidence countries like India. Aim of the study was to access and compare sensitivity and specificity of Light emitting Diode Fluorescent Microscopy (LED FM) over Light microscopy (LM) for diagnosis of pulmonary (PTB) and extra pulmonary tuberculosis (EPTB).

Methods: 1446 pulmonary and 328 extra pulmonary cases suspected of tuberculosis were included in the study. Sputum samples were collected on 2 successive days while only one EPTB specimen was received in the laboratory.

Results: Out of 2767 sputum specimen and 328 EPTB specimen evaluated, 603 (21.79%) sputum and 16 (4.84%) EPTB respectively were grown on culture and considered positive for Mycobacterium Tuberculosis. The sensitivity for sputum samples from ZN stain direct smear and concentration sputum smear by LM was 57.88% and 67% respectively, while the specificity of both direct and concentration smears by LM ZN staining was 100%. In case of sputum samples sensitivity of LED FM has drastically increased to 82.09% and the specificity is only slightly lower than LM that is 99.88%. For EPTB sensitivity for direct smear by LM was found to be 12.50% and concentration smear was 18.75% with 100% specificity. With LED FM sensitivity was enhanced by 43.75% with only marginally less specificity as 99.67%. All differences between culture and smear positive were highly statistically significant. (P value <0.0001).

Conclusion: LED FM has higher sensitivity and almost similar specificity (p value <0.0001). Since it has many beneficial attributes it can be a certain substitute to light microscopy and can improve and decentralize diagnostic services, especially in low and middle income countries (LMIC). Smear microscopy services should be optimized and can be used along with the add on test, to many new diagnostic approaches like cartridge based nucleic acid amplification test (CBNAAT) MTB/RIF GeneXpert.

Keywords: Extra pulmonary tuberculosis (EPTB), LED Fluorescence microscopy, light microscopy (LM), pulmonary Tuberculosis (PTB), Sensitivity, Specificity.

Access this article online
Quick Response Code:
Website: www.innovativepublication.com
DOI: 10.5958/2394-5478.2016.00069.8

Introduction

Tuberculosis (TB) continues to daunt the humanity ceaselessly as a severely debilitating disease. In resource limited settings and in low and middle income countries (LMICs), diagnosis of tuberculosis reckons mainly upon smear microscopy that is Ziehl Neelsen (ZN). Compared to light microscopy (LM), fluorescence microscopy (LED FM) has various potential advantages. The socio-economic impact of TB captivates implications for the major efforts that are underway to venture and achieve its control. Worldwide, 9.6 million people are estimated to have fallen ill with TB in 2014. Globally, 12% of 9.6 million new TB cases in 2014 were HIV positive.[1] To reduce this burden, detection and treatment gap must be addressed, funding gaps closed and new tools must be developed.[1]

Despite of the long established tradition of solid culture for mycobacterium tuberculosis and numerous more recent advances in tuberculosis diagnosis are leading to introduction of new test, like liquid culture, line probe assay (LPA) and Cartridge Based Nucleic Acid Amplification Test (CBNAAT). As per RNTCP guidelines in India, microscopy forms the mainstay tool in the management of TB. RNTCP has also prioritized decentralization of treatment services, so that the furtherance are accessible and acceptable to the patients. If we enhance the sensitivity of smear microscopy definitely more cases of TB can be diagnosed early. With this prior knowledge, improvisation are made to upgrade the microscopy techniques and ameliorate the procedure of specimen collection and its processing.[2,3,4]

The number of mycobacteria to be detected by microscopy should be in between 10^4 and 10^5 bacilli per ml of sputum. Under the national program conditions the rate of detection of AFB is often reduced due to technical and operational constrain.[5] ZN staining is highly specific for detection of TB bacilli by Light microscopy, but sensitivity of this technique is quite variable (20 to 80%) in patients with HIV TB, extra pulmonary TB, and paediatric population where the sensitivity is significantly further reduced.[6] Auramine Staining that can be visualized by fluorescence microscopy (FM) has

the sensitivity higher by 10% compared to Ziehl Neelsen staining seen by Light Microscopy without compromising specificity.[7] Less time is required to review the smears stained with Auramine O, one study has reported that FM takes only 25% of time required for ZN examination.[8] Light emitting diode (LED) microscopes are cheap, smears can be scrutinized under high power instead of oil emulsion lens and since it can run on batteries it can be used in periphery and PHCs where cut off of power supply is a big problem furthermore, the bulb has the long half-life and is safe compared to conventional FM which often releases substantially toxic product if broken down, and LED FM does not require dark room unlike conventional mercury light source.[9,10]

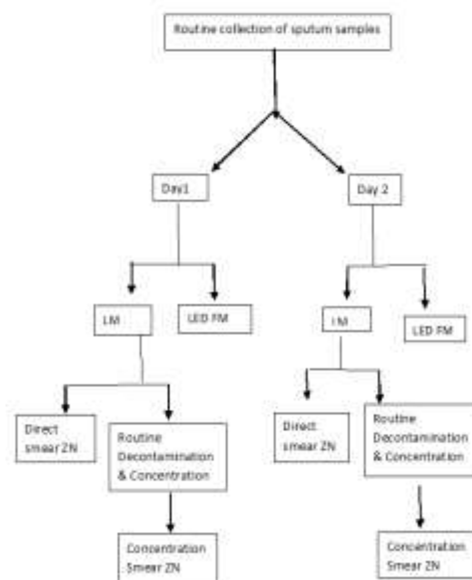
The result of sputum AFB microscopy is known to be influenced by various factors, including the proficiency to read the smear by microscopist. Quality of examining AFB smears, needs to be ensured by training the laboratory technicians (especially in case of FM), and by ensuring that the smears are screened for approved duration of at least 5 min or 100 fields for Light microscopy could be the simple and low-cost way to improve case detection.[11,12]

Methods

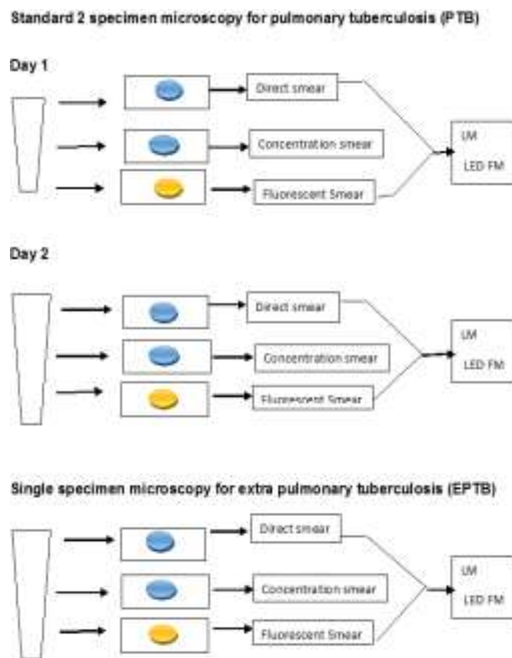
Specimen collection and evaluation procedures: We conducted a cross sectional, laboratory-based study. A total of 2892 specimen of sputum were collected from 1446 patients from July 2015 to march 2016 and referred to the laboratory by our tertiary care hospital. Standard assessment of patient included early morning sputum specimen collection on two consecutive days in order to perform acid fast bacilli (AFB) smear microscopy and culture. The patient who had submitted only one specimen where not included in the study, laboratory technicians provided standardized instruction on proper sputum submission to the patients.[13]

We also received 328 extra pulmonary specimen in our laboratory which included 28 tissue, 54 pleural effusion, 25 bronco-alveolar lavage (BAL), 12 tracheal secretions, 44 pus, 18 cerebro-spinal fluid (CSF), 27 gastric lavage (GL), 12 ascitic fluid, 66 Lymph node FNAC, 33 urine and 9 peritoneal fluid. All pulmonary and extra pulmonary specimens were decontaminated and concentrated using N acetyl L cysteine sodium hydroxide (NALC NAOH) procedure except for CSF.[14] We processed all the specimen using the conventional centrifugation method in a refrigerated centrifuge (Flowchart 1).

Flowchart 1: An overview of specimen assessment and evaluation. Note: For all extra pulmonary samples same protocol has been employed but only one sample was received and processed.



AFB Smear Microscopy: Technician prepared total of 3 smears (Flowchart 2) for both LM and LED FM. From each specimen for LM: 2 smears were prepared, one direct smear and one smear from concentrated specimen after routine decontamination and concentration by NALC NAOH method. These smears were then subjected to ZN staining by carbolfuchsin (Fig. 1). The grading was done as per RNTCP guidelines.[15] For LED FM: one separate smear was made from each specimen and stained with Auramine-O (fluorochrome) stain. Mycobacteria appeared as bright yellow fluorescent rod in direct smear with fluorochrome staining, when viewed under excitatory light source (Fig. 2) and grading of all the pulmonary and extra pulmonary specimens for fluorochrome smears was done according to IUATLD guidelines.[16]



Flowchart 2: Smear microscopy strategies: Standard approach to smear microscopy for sputum specimen involves collection of sputum over 2 consecutive days and two smears were prepared from each specimen for LM stained by ZN staining that is direct and concentration smear respectively. One direct smear was made for LED FM and stained by auramine o stain (Top panel). Single specimen microscopy was done for EPTB samples in the similar way (bottom panel). With both strategies smears were examined using both conventional LM and Light emitting diode (LED) FM.

Mycobacterial Culture: Every pulmonary and extra pulmonary specimen sediments were inoculated on to the slope of plain Lowenstein Jensen (LJ) medium as well as slope of LJ medium containing PNB (p-nitrobenzoate) and was incubated at 37^0C in 5 % CO_2 for up to 8 weeks for smear negative samples and up to 12 weeks for smear positive samples. The gold standard or reference method used was culture and was compared with the ZN and FM techniques. We interpreted, the patient as positive for TB, if Mycobacterium tuberculosis was isolated from any pulmonary and extra pulmonary specimen and absent if **(1)** Isolation of Mycobacterium tuberculosis was not seen in any pulmonary and extra pulmonary sample and **(2)** Negative sputum culture, at least one (i.e., not contaminated)

Results

Of the 2767 sputum specimens and 328 EPTB specimens evaluated, 603 (21.79%) sputum and 16 (4.89%) EPTB samples respectively were confirmed by culture to be positive for mycobacterium tuberculosis. For PTB, LM, ZN staining, identified 349 (12.61%) direct smear, the sensitivity been 57.88% and specificity was 100% with the 95 % confidence interval of 53.82% to 61.85% and 99.80% to 100% respectively. And 404 (14.60%) smears were positive by LM after routine decontamination & concentration by NALC NAOH method and sensitivity of the concentration smear was increased to 67% with 95% of confidence interval (CI) of 63.09% to 70.74%, specificity of the concentration smears were 100% with 95% CI of 99.80 to 100%. For fluorochrome staining, 495 (17.89%) sputum smears were positive for AFB so the sensitivity of FM is highest amongst the 3 modalities of microscopy that is 82.09% with 95% CI of 78.79% to 85.07% and since 2 FM smears were false positive specificity is slightly lower than LM, ZN staining method that is 99.88% with 95% CI of 99.57% to 99.99% (Table1 and 2). The increase in yield examining more than one sample was very similar for all microscopy strategy. All the differences between yields of smears verses cultures were highly statistically significant ($P<0.0001$). Considering all microscopy results (excluding those from specimen with contaminated culture), for sputum specimen, on LM, concentration smears diagnosed 55 more and LED FM diagnosed 146 more sputum to be smear positive compared to the direct smear from the specimen stained by ZN.

All extra pulmonary specimen are known to be paucibacillary. Among 328 EPTB specimens received 16 (4.88%) were culture positive. In LM by ZN staining the direct smear of only 2 (0.61%) specimen out of 328 were positive, so the sensitivity is only 12.50% with 95% CI of 1.55 to 38.35% and the specificity is 100%. 3 of the 328 (0.91%) specimen were positive in concentration smears with the sensitivity of 18.75% and 95% CI of 4.05% to 45.65%, specificity is 100%. Similarly 7 of the 328 (2.13%) smears were positive by LED FM so the sensitivity has been increased to 43.75% with 95% CI of 19.75 to 70.12% and only one false positive was detected by FM so the specificity noted was 99.67% with 95% CI of 98.19 to 99.99% (Table 3 and 4). All differences between culture and smear positives (including the individual technique) were highly statistically significant ($P<0.0001$). Extra pulmonary specimen received in the lab included 28 samples for tissue culture out of which none was positive for microscopy and culture. 54 Pleural effusion samples were received of which 4 samples were culture positive and none were positive for either culture or microscopy, 25 BAL were received 3 were positive by culture and 1 BAL was positive by all the microscopic modalities, 44 pus samples were received only 2 were positive by culture 1 by concentration smear and 2 by auramine O Staining, 18 CSF and 27 GL were referred in laboratory 1 each was positive by culture and none by microscopy, out of 66 LN FNAC received 5 were positive by culture and one FNAC sample was positive for AFB by LM (both direct and concentration smear), and LED FM could detect 4 samples to be positive for smear microscopy, while 12 ascitic fluid, 33 urine, and 9 peritoneal fluid were also received of which none is

positive for microscopy as well as culture. For both pulmonary and extra pulmonary specimen the yield achieved with LED FM exceeded the yield achieved with LM. Concentration technique improved the detection rate of AFB compared to direct smear by LM. Considering all microscopy results (excluding those from specimen with contaminated culture), for EPTB specimens LED FM diagnosed extra 4 specimen to be smears positive than LM which were scanty according to IUATLD grading system used for FM.[16] The contamination rate in our study was found to be 2.77%.

Table 1: Comparison of patient wise result in Ziehl-Neelsen (ZN) and fluorochrome microscopy (FM), by culture status (patients with all culture contaminated excluded) for PTB

	Culture (%)	ZN Staining Direct Smears (%)	ZN Staining Concentration Smears (%)	Fluorochrome Staining Direct Smears (%)
Positive	603 (21.79)	349 (12.61)	404 (14.60)	495 (17.89)
Negative	2164	2418	2363	2272
Total No of specimen	2767	2767	2767	2767

Table 2: **Sensitivity, Specificity, Positive and Negative Predictive Value of different methods of staining for PTB**

Sputum n = 2767	Culture positive (CP) n= 603	Sensitivity	Specificity	PPV	NPV
ZN Direct	349 (12.61%)	57.88%	100%	100%	87.72%
ZN Concentration	404 (14.60%)	67%	100%	100%	89.84%
FM Direct	495 (17.89%)	82.09%	99.88%	99.60%	93.92%

PPV = Positive Predictive value, NPV= Negative predictive value.

Table 3: Comparison of patient wise result in Ziehl-Neelsen (ZN) and fluorochrome microscopy (FM), by culture status (patients with all culture contaminated excluded) for EPTB

	Culture (%)	ZN Staining Direct Smears (%)	ZN Staining Concentration Smears (%)	Fluorochrome Staining Direct Smears (%)
Positive	16 (4.88)	2 (0.61)	3 (0.91)	7 (2.13)
Negative	312	326	325	321
Total No of specimen	328	328	328	328

Table 4: Sensitivity, Specificity, Positive and Negative Predictive Value of different methods of staining for EPTB

EPTB n =328	Culture positive n= 16	Sensitivity	Specificity	PPV	NPV
ZN Direct	2 (0.61%)	12.50%	100%	100%	95.71%
ZN Concentration	3 (0.91%)	18.75%	100%	100%	95.99%
FM Direct	7 (2.13%)	43.75%	99.67%	87.50%	97.13%

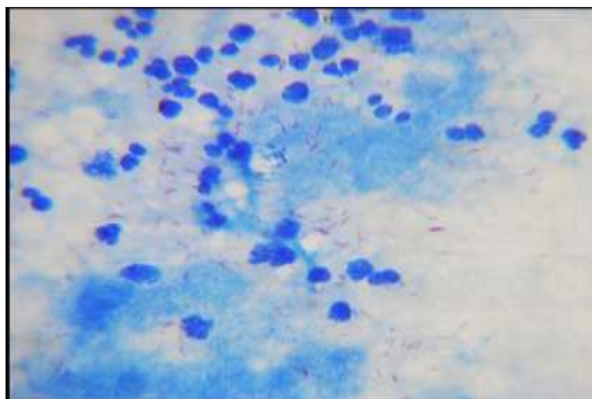


Fig. 1: Ziehl Neelsen Microscopy: Mycobacteria appears as bright red under Light Microscopy (X 1000)

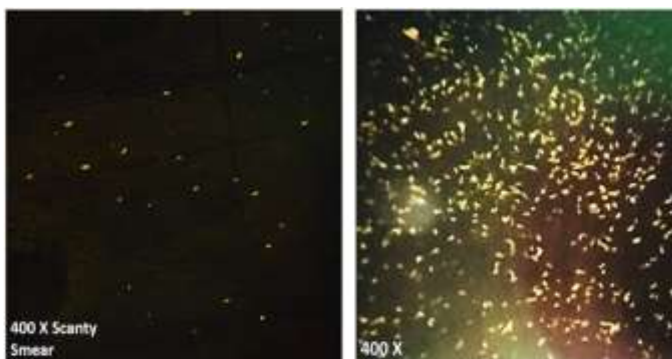


Fig. 2: Fluorochrome Microscopy: Mycobacteria appears as bright yellow fluorescent rod when viewed under excitatory light source (X 400)

Discussion

For global TB control it is an urgent priority to develop strategies to improve efficiency and sensitivity of smear microscopy. In absence of simple, low cost alternatives to sputum smear microscopy much effort has been expanded in recent years in developing optimized smear microscopy procedures.[17] In our study sensitivity of LED FM direct smear is much more superior to LM, ZN staining from both direct and concentration smears, with almost similar specificity (99.88%). Our result suggest that smear positive pulmonary and extra pulmonary TB can be detected early in low income and high TB burden countries by LED FM either alone or in combination. The light source of LED FM requires less energy and is battery operated hence it is useful for places where power supply is uncertain and fluctuating as it is seen frequently in remote areas and or resource limited settings.

As time required for screening the smears by fluorescent microscopy is less and excellent sensitivity and specificity is achieved by FM, it should be made readily accessible especially in resource limited setting with a high burden of TB. Time saving attained by FM is attributed to quicker scanning of each field because of increased visibility of mycobacteria; and is not due to reduction of number of fields screened (100 fields were screened with both modalities). In FM, low magnification is used, (X 200 and X 400) and in LM higher magnification is used (X 1000), to screen the smears this could be a reason towards sensitivity difference noted. Auramine O staining method is much simpler than the ZN staining method which could be an additional benefit of fluorochrome staining.[7,18]. Introduction of LED FM devices in to national TB control programme may have to make allowance for possibility that their performance could vary with the experience of faculty, basic infrastructure of the laboratory and the type of devices used. The performance of LED FM, and indeed any diagnostic test, depends critically on proficiency of test operators. So adequate training needs to be implicated to make the staff, an adept, competent and masterful, for screening the smears by LED FM. To our experience laboratory technicians those who were already proficient in ZN smear microscopy do not require much training to expertise the LED FM. Training of the staff regarding microscopy included not only staining techniques and supervision of routine microscopy but also included the instructions and supervision regarding specimen processing which indeed helped resolve any doubt and instil confidence in the workers. All personnel expressed their acquaintanceship and steadiness in their capability and handiness to perform the technique. We opine that for the continuous mentoring process for improving performance and competence in staff, this kind of training is sufficient enough. Being an ISO 15189 (2012)

NABL (National Accreditation for testing and calibrating laboratories) accredited laboratory the quality of ZN and FM is assured by having an effective inter laboratory comparison (ILC) programme in place. The LED FM admittance needs to be abetted looking at the performance indicator, careful training and relevant quality control.

Moreover the LED FM distinctive role needs to be pondered even though WHO had endorsed new rapid, automated cartridge based nucleic acid amplification test, Xpert. Even though the smear microscopy can be replaced by Genexpert as the initial diagnostic tool worldwide, decentralization and scaling up of xpert sufficiently enough in short duration is doubtful. Smear microscopy used as primary investigation, is also favoured because of its cost effectiveness and can be used in areas with low rates of HIV associated TB or multi-drug resistant TB. The enhanced role of direct smear microscopy indeed the technique like LED FM which is much faster than the conventional LM, improving diagnostic services that would reduce the laboratory workload and which would detect the smear negative patient who could undergo further test. Moreover there is barely any difference in the specificity of LED FM (99.88%) and ZN LM (100%) in our study which elects it to an operational advantage and an attractive tool for laboratory diagnosis of PTB and EPTB. The commencement of LED-FM in to the National control programs needs the monitoring of the conditions in the laboratory because the requirement for training the faculty could be higher than intended. Study reveals and elucidates on the crucial role of LED FM in early detection and prompt treatment, reducing work load of the laboratory by enhancing faster detection and because of its battery backup, clinching poor patients' access to diagnostic services.

Conclusion

It is utmost important, to optimize the smear microscopy services to provide early diagnosis prompt treatment to the patients in resource constraint and poor countries. While the MTB/RIF and other diagnostic tools are piled out, this rolling may take several years before adequate service coverage is achieved and also to provide enhanced performance of diagnostic services in the areas where the new diagnostic test such as genexpert are being considered as an add on test to sputum smear microscopy for treatment monitoring. It will also minister the best possible baseline data to judge the performance and added value of new technologies as they are introduced.

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How to cite this article: Rathod UD, Joshi A, Turbadkar S, Hirani N, Chawdhary A. Different Methods of Staining to optimize Smear Microscopy for Diagnosis of Pulmonary and Extra Pulmonary Tuberculosis. Indian J Microbiol Res 2016;3(3):322-328.