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Viable Staining for Detecting Mycobacterium Pulmonary Tuberculosis in Vulnerable Population

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Abstract

Background: Diagnosis of pulmonary tuberculosis (PTB) is the major concern of proper diagnosis and for effective treatment of PTB. Despite the recent advancement in diagnostic methods, the smear microscopy remains the gold standard for the diagnosis of PTB in high burden countries. **Design:** This study, therefore, aimed at assessing the diagnostic performance of fluorescein diacetate (FDA) and Ziehl–Neelsen (ZN) staining techniques in the diagnosis of PTB for vulnerable group of population HIV, smoker, health care workers, and malnutrition people and confirmed with Mycobacterium Growth Indicator Tube (MGIT) liquid culture to check the viability. Samples were collected from Intermediate Reference Laboratory, Karnataka, from November 2019 to March 2020. A total of 350 samples were collected and these were stained using ZN method as per National Tuberculosis Elimination Program norms and FDA method followed by liquid culture using the MGIT 960 to check for the viability of the bacilli and correlate with the results of the FDA viability staining method. **Results:** The study demonstrates that the sensitivity and specificity of FDA viable fluorescent staining to that of liquid MGIT culture were 98.6% and 100%, respectively, while those of ZN staining were 55.8% and 100%, respectively. **Conclusion:** Hence, we concluded that the FDA staining technique is a more sensitive test for checking the viability of AFB among PTB suspect, as compared to the conventional ZN-stained smear observation.

Keywords: AFB, Fluorescein diacetate, Mycobacterium Growth Indicator Tube liquid culture, Pulmonary tuberculosis, Ziehl–Neelsen

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Introduction

Tuberculosis caused by the tubercle bacillus remains a significant public health issue with approximately one-third of the world's population being affected by it. In 2017, 10 million people were infected with tuberculosis and 1.6 million died from the disease. Over 95% of tuberculosis deaths occur in low- and middle-income countries.[1] A faster, simpler, more accurate, and less expensive means of diagnosis of tuberculosis are imperative for the control of infection and to cut the chain of its transmission within the community.[2] Various investigations are often used to help with the diagnosis of tuberculosis, which includes chest radiographs, clinical presentation of the patient, culture for mycobacteria, macromolecule amplification assays, smear staining for acid-fast bacilli, and microscopy.

Smear microscopy is the most preferred and rapid diagnostic tool that is widely used for the detection of the disease.[2,3] The bacilli within the sputum are often detected either by Ziehl–Neelsen (ZN) or fluorescence staining techniques. Sputum microscopy is useful to assess the patient's response to treatment. In many developing countries, diagnosis of tuberculosis is supported by ZN staining and microscopy.[4] Sensitivity of smear microscopy by ZN staining, however, is reported to be low and variable, ranging from 20% to 80%, often depending on the diligence with which the specimens are collected, smears prepared, and examined.[4-7] This procedure leaves a large number of cases undetected, especially if it is the sole means of diagnosis.

The fluorescein diacetate (FDA) vital staining technique helps to boost the outcomes of smear microscopy. FDA staining technique works on the principle of intracellular hydrolysis of FDA. Just living cells effectively convert the non-fluorescent FDA into the green, fluorescent compound fluorescein following enzymatic movement, which is viewed as an indication of feasibility. A smear is defined as FDA positive if at least one fluorescent bacillus per 100 high-power fields is observed. The sensitivity of FDA staining provides better detection of positive smears than the ZN staining method and is less time consuming.[8-10] Despite all the above conveniences, the cost constraint is the only limitation for deployment of the FDA stain microscopy, especially in low- and medium-income resource-limited settings or in the peripheral microscopy centers.

In the State of Karnataka, India, the diagnosis of pulmonary tuberculosis (PTB) using conventional smear microscopy by ZN staining is gradually being replaced by microscopy of smears stained using fluorescent stains (fluorescent microscopy [FM]). Use of most approved molecular techniques requires large laboratory resources and dedicated staff. There is currently no documentation or evidence on the application of FDA-stained smear microscopy, for the diagnosis of PTB. Hence, this study compares the three methods, namely, ZN stained, fluorescent dye stained, and FDA-stained smears by microscopy for the detection of PTB.

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Materials and Methods

Sample source

This study was carried out, in the Intermediate Reference Laboratory (IRL), at NIMHANS, Bengaluru, Karnataka, India. The laboratory has been accredited since 2010, by the National Mycobacteriology Accreditation System of the Central TB Division, Ministry of Health and Family Welfare, Government of India. All pulmonary samples received at the IRL, from tertiary primary health-care centers following a positive ZN smear microscopy result, were registered for confirmation and further analyses at IRL, Bengaluru, Karnataka, for this study (Table 1 and Chart 1).

Sample size and sampling period

All pulmonary specimens (sputum specimens) collected from the TB patients and received at the IRL at SDS Sanatorium, NIMHANS, Bengaluru, for a period of 4 months (November 2019–March 2020), from various districts in the State of Karnataka for reconfirmation of positive status and further culture was included in the study. Sputum samples were received in wide-mouthed sterile 50 ml falcon tubes. A total of 350 sputum samples from smear-positive PTB patients (according to the acceptance criteria of National Tuberculosis Elimination Program [NTEP], India, earlier the Revised National TB Control Programme) were taken up. Smears were prepared in duplicates per sample to check for smear positivity by the ZN staining method as per NTEP guidelines (Table 2). The FDA stain preparation, staining, and microscopy were performed as per Tsukiyama *et al*. and Kanade *et al*.[11,12] to check for viability of the cells (Table 3).

The remaining sample was used to set up culture for *Mycobacterium tuberculosis* on solid and liquid culture media. Decontamination of the sample was done as per the standard operating procedures prescribed by the NTEP, using the *N*-acetyl-$_L$- cysteine-sodium hydroxide method. One culture tube for incubation in the automated Mycobacterium Growth

Table 1: Category of samples

Vulnerable group	*Samples*
HIV	134
Smokers	75
Health care workers	25
Malnutrition	116
Total	350

Table 2: ZN grading chart

Result (WHO scale) 1000×field=HPF	*Bright fields×1000: 1 length=2 cm=100 HPF*
Negative	Zero AFB/100 HPF
Scanty	1–9 AFB/100HPF
1+	10–99 AFB/100HPF
2+	1–10 AFB/1HPF (on average 50 HPF)
3+	>10 AFB/1 HPF (on average 20 HPF)

ZN: Ziehl–Neelsen

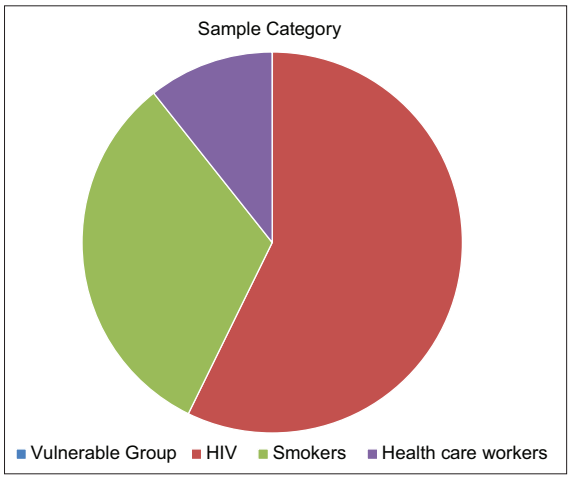

Chart 1: Sample Category according to Vulnerable group

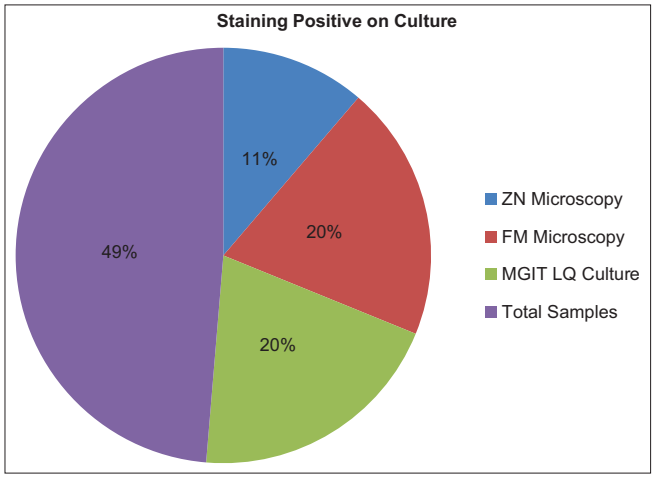

Chart 2: Comparison of staining with culture

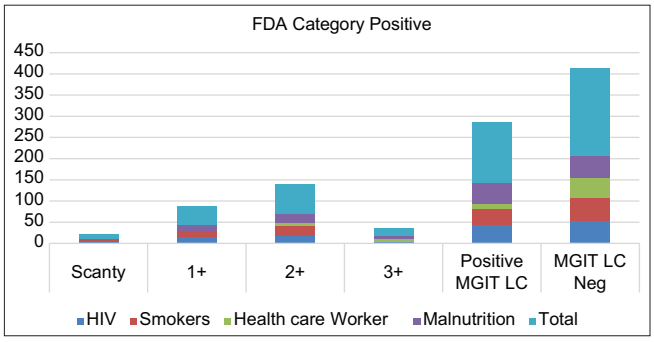

Chart 3: Fluorescein diacetate positive in different vulnerable group with LC

Table 3: Grading chart (WHO, IUATLD, 2007) for led fluorescent microscopy

Result (WHO scale)	*LED fluorescent*	*Minimum number of fields to be examined*
Negative	Zero AFB/1 length	40
Scanty	1–19 AFB/length	40
1+	20–199 AFB/1 length	40
2+	5–50 AFB/1 field on average	20
3+	>50 AFB/1 field on average	8

Indicator Tube (MGIT) 960 liquid culture system and two tubes with the solid egg-based Lowenstein-Jensen culture medium were inoculated per specimen. Smears from the positive cultures were observed microscopically following FDA staining, ZN staining, and identification of the culture as *M. tuberculosis* using the rapid cartridge-based nucleic acid amplification test along with the lateral flow immunochromatography test MPT 64 (Capilia TB test) for the differentiation of *M. tuberculosis* complex from NTM. If viable mycobacteria are present in a specimen, they will grow in the MGIT liquid culture medium and will be detected visually as well as by emitting fluorescence with FDA staining microscopy. Results were reported only when a MGIT tube is positive by the instrument and smear made from the positive culture is also positive for FDA. If all the three media tubes on solid and liquid culture were found to be contaminated, the sample was considered "contaminated." A culture was classified as "*M. tuberculosis* positive" only if the rapid identification test MPT 64 Antigen (Capilia TB test) for all three culture tubes inoculated on solid and liquid culture was positive. Contaminated cultures were excluded from the performance analysis.

Results

In the 350 samples analyzed, 143 (40.85%) in FDA viable staining, 81 (23.1%) in ZN microscopy, and 145 (41.4%) were positive in MGIT liquid culture, respectively (Table 4 and Chart 2). The 143 FDA staining positive samples at different vulnerable population was found to be on HIV 43 (30%), smokers 39 (27%), Health care workers 13 (9.09%), Malnutrition 48 (33.5%) respectively (Table 5 and Chart 3) while the mean reading time of FDA staining Fluorescent Microscopy was 3 times faster than the ZN technique with a good acceptance rate of (1.5min: 4.6min). The sensitivity and specificity of FDA viable fluorescent staining to it of liquid MGIT assay were 98.6% and 100%, respectively, while those of ZN staining were 55.8% and 100%, respectively.

Discussion

Tuberculosis may well be a significant public unhealthiness everywhere the country to keep with ages, with such plenty of advances in treatment and management, still tuberculosis can be a public problem in India with adverse social and economic conditions.[13] Current recommendations for the control of tuberculosis emphasize early case detection, treatment of patients, and thereby limit the transmission of the bacilli.[14] The mainstay for its control is that the rapid and accurate identification of the infected individuals..[15] The detection of AFB is taken under consideration because the evidence of infective stage. The research facility assumes a basic part in determination of utilization of high complex gear of other symptomatic tests that utilization of molecular and immunological strategies are created. While molecular techniques defeat the heartlessness of smear strategy, the time needed for culture and recovery of a specimen from the situating of disease require good to go research facility and all around prepared staff the best quick strategy is that the recognition of acid-fast bacilli by microscopy. In the developing nations, microscopy of sputum is a lot and away the quickest, least expensive, and more dependable technique for analysis of utilization. The estimated detection limit of microscopy is 10^7 bacilli/ml of sputum.[16] In immunocompromised patients like HIV affected cases, there is a significant effect on the pathogenesis of tuberculosis, it straight forwardly assaults the basic resistant systems associated with assurance against tuberculosis. ZN stain can recognize bacilli which when put on to culture were seen negligible inspiration, whereas a more delicate auramine stain can distinguish just bacilli which can develop on to culture.[17] For that reason, there is a necessity for few more rapid and accurate tests with spending limited time and be able to carry out easily at a resource limited conditions. Hence, we endeavored to assess FDA vital staining process in the detection of viable AFB and these results were compared to that of liquid MGIT assay. FDA is a salt of fluorophore, such as fluorescein, and it has the ability to penetrate the viable mycobacteria through hydrophobic cell membrane and get hastily hydrolyzed by esterase's present in the cells. This event takes place only within the viable cells and it facilitates to fluoresce under LED fluorescent microscope and on the contrary the dead AFB lacks this activity and not able to fluoresce.[18]

In our present investigation, out of 350 samples examined, 143 (40.85%) and 81 (23.1%) TB cases were positive for FDA viable staining and ZN staining methods, and 207 (59.15%) and 269 (76.9%) were negative for those tests, respectively. Where the sensitivity and specificity of fluorescent staining there to be of liquid MGIT assay were 98.6% and 100%, respectively, while those of ZN staining were 55.8% and 100%, respectively. Similarly, the study was conducted by Kanade *et al.* (2016), using the sputum samples of either Cat I or Cat II anti-TB treatment reported that, of the 100 ZN-positive specimens, 74 were positive for FDA of which 70 were reported positive by both the readers. They also reported that MTB was isolated in 75 specimens of which 72 were FDA positive and two culture-negative samples were also positive by FDA. The sensitivity and specificity of FDA staining in previous study by Kanade, S., Nataraj, G., Ubale, M., & Mehta, P, correspondingly was 96% and 92%.[12]

Recently, Ahmed *et al.* (2019) showed the sensitivity (84.93%), specificity (95.74%), positivity (96. 50%), and negativity value

Table 4: Comparison of FDA viable staining with ZN staining and culture

Methods	*Positive (%)*	*Negative (%)*
ZN microscopy	81 (23.1)	269 (76.9)
FDA	143 (40.85)	207 (59.15)
MGIT LQ culture	145 (43.4)	205 (56.6)
Total samples	350	

ZN: Ziehl–Neelsen, MGIT: Mycobacterium Growth Indicator Tube

Table 5: FDA positive on MGIT LC

FDA staining	*Scanty*	*1+*	*2+*	*3+*	*MGIT LC (+ve)*	*MGIT LC (–ve)*
HIV	5	15	18	5	43	53
Smokers	3	13	23	0	39	56
Health care worker	0	0	8	5	13	45
Malnutrition	3	16	21	8	48	53
Total	11	44	70	18	143	207

(+ve): Positive; (–ve): Negative. MGIT: Mycobacterium Growth Indicator Tube

82.12% of ZN staining technique whereas in FM was recorded as 95.25% sensitivity, 91.33% specificity, 93.81% positivity, and negativity value 93.31%. They also reported that compared to ZN staining, AO staining LED-FM was 7.48% more effective.[19] Another study conducted by Shuudeni (2021) with 142 smear-positive samples, reported the sensitivity and specificity of FDA staining to be 99.14% and 96.43% and the positive predictive value to be 99.14% whereas the negative predictive value was 96.43% and concluded that the accuracy of FDA in their study was found to be 98.61%.[20] Even though it has high sensitivity and specificity, FDA staining has few minor limitations such as the frozen of stock solutions at −20°C and the need for freshly prepared working solutions on every occasion. Furthermore, there is a chance of fading of smear as it does not examine at earlier. Likewise, this technique has an advantage such as reagents cost is similar to other technique like fluorescent staining and thus this technique can be used in peripheral diagnostic centers having the facility of LED microscopy.

Conclusion

Sputum assessment for the tubercle bacilli is ordinarily directed for patients clinically and radio intelligently associated with tuberculosis disease. Nonetheless, the normal strategy for sputum assessment, that is, ZN staining is not adequately touchy and same presumed cases are not affirmed. Moreover, they keep on being undiscovered and neglect to ask treatment. Consequently, our review presumes that fluorochrome staining with LED is more effective over ZN stain in recognizing tuberculosis bacilli in sputum, particularly the paucibacillary cases, and furthermore, FDA has been figured out to be less tedious when contrasted with ZN strategy (1000×) inside the determination of TB. FDA staining with LED is easier to utilize, speedier, and less expensive particularly in focuses where enormous quantities of sputum examples are handled. The sole inconvenience with fluorescent staining is that the worth of LED magnifying instrument is nearly high and won't be reasonable for developing nations. At whatever point, it is conceivable, cytosmear assessment ought to be finished by fluorescent strategy by progressively supplanting the ordinary magnifying instruments with LED fluorescent ones.

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