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Evaluation Of Fluorescein Diacetate (FDA) Vital Staining In Detecting Viable Acid Fast Bacilli In Comparison With Culture In A Tertiary Care Hospital

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ABSTRACT

Background: Peripheral laboratories in developing countries usually lack the facilities of culture and drug susceptibility testing and mostly relay on smear microscopy which is used as a basic tool in diagnosis of Tuberculosis. But the routinely used microscopic techniques such as ZN staining and Fluorescent microscopy cannot discriminate the live and dead bacilli.

Objectives: Hence, this study is aimed to evaluate Fluorescein diacetate (FDA) vital staining technique in detection of viable acid fast bacilli among smear positive sputum samples from a tertiary care hospital situated in Mysuru, South India.

Results: A total of 142 smear positive sputum samples were subjected to Auramine staining, FDA staining and Culture on LJ-Media . Considering culture as gold standard, FDA staining had a sensitivity of 99.14% and specificity of 96.43%. Positive predictive value, Negative predictive value of FDA staining was 99.14 and 96.43% respectively. Diagnostic accuracy of FDA staining in our study was found to be 98.61%.

Conclusion: Implementation of FDA staining in peripheral laboratories with limited access to culture and drug susceptibility testing would help in minimizing the culture and drug susceptibility testing load in referral centres.

Keywords:
Vital Staining, Acid fast bacilli, culture

1. Introduction

Tuberculosis, a disease of public health importance in the 21st century and multidrug resistant TB has amplified the disease burden. In 2018, the estimated proportion of MDR/RR-TB is 2.8% among new cases and 14% among previously treated cases. An average 199 patients per 1 lakh population were notified with TBof which only 57% were bacteriologically confirmed and rest did not have microbiological confirmation of diagnosis[1]. Limited access to diagnostic tests, poor performance of testing the diagnostic samples and other multifactorial reasons may be responsible for the above reasons[2,3]. This epidemic can be controlled only with early detection of TB patients and execution of anti-TB treatment (ATT) at the earliest. A minimum of 6 months of treatment including 2 months of intensive phase (IP) and 4 months of continuation phase (CP) is required for the treatment of TB patients. Follow upat regular intervals is necessary during the course of treatment, which would help in monitoring the treatment response and for early detection of drug resistance. Culture is always considered as the gold standard diagnostic method for TB and for definitive determination of drug resistance. Culture has a capability in identifying paucibacillary disease and can discriminate live and dead bacilli too unlike other conventional microscopic methods and PCR techniques. But solid culture of MTB on LJ-media takes several weeks to obtain the results. Introduction of automated liquid cultures into the diagnosis of TB such as MGIT 960, has reduced the turnaround time to 2-3 weeks. These solid, liquid cultures and other PCR based techniques require high level of expertise and sophisticated laboratory infrastructure.

Peripheral health centres (PHC's) and other resource limited settings usually lack such laboratory infrastructure and those centres rely on microscopic examination of the follow-up specimens, which is the only tool that is available for diagnosis and monitoring the treatment response among TB patients. Follow-up specimens from TB patients are usually examined after the completion of 2 months of intensive phase and once again after 4 months of continuation phase[3]. Few studies have documented different ranges of sputum conversion at the end of IP. In a study conducted by H.L. Rieder, sputum conversion by microscopy after 2 months of IP was 75%[4]. A positive follow-up smear microscopy can occur due to the delay in smear conversion rate after the IP or CP, this can result in unnecessary prolongation of ATT and an extra burden for National Tuberculosis Elimination Programme (NTEP). Few studies have already proposed the use of Fluorescien diacetate (FDA) as a viability staining technique[5] which to be used in combination with smear microscopy[6,7]. This can be a potential tool for monitoring the treatment response in resource limited settings. This technique based on the principle of intracellular FDA hydrolysis and generally causes viable cells to fluoresce. Only living cells actively convert the non-fluorescent FDA into the green fluorescent compound following enzymatic activity i.e the hydrolysis of stain in the cytoplasm, happens only in the presence of non-specific esterase in metabolically active cells[8]. Presence of fluorescent bacilli in the follow up clinical specimens indicates drug resistant strains. Hence, this study was aimed to evaluate Fluorescien diacetate (FDA) vital staining technique in detection of viable acid fast bacilli among smear positive sputum samples and comparison of FDA vital staining results considering culture as gold standard.

2.Materials and Methods:

This is a laboratory based prospective study conducted in the department of Microbiology, JSS Medical college and Hospital, Mysore, India. The study was started after obtaining Institutional ethics committee clearance. Treatment naïve patients and patients either on IP or CP treatment submitting their sputum samples for diagnosis or follow-up were included in the study. Repeat samples from the same patients, insufficient quantity of samples and samples containing saliva were excluded from the study. As this study involved the samples that are received in the laboratory for routine diagnosis, and the patients are not directly involved, Informed consent form was not taken from the patients. All the samples were subjected to Microscopic examination using Auramine O staining technique, FDA staining and cultured onto LJ-Media for the isolation of Mycobacteria.

Briefly, smear was made from the mucous portion of the sputum sample, heat fixed and the stained with Auramine O solution for 7 minutes, washed under tap water, decolourized with 0.5% acid alcohol for 3 minutes. After washing the smear was counterstained with 0.5% $KMNO_4$ and was be observed under the LED micro Zeiss (Heidenheim, Germany) iLED microscope for the presence of AFB.

Those samples which are positive through Auramine staining technique are also stained using FDA staining technique for detection of viable AFB. FDA staining of the smears was performed as described by Tsukiyama et al[9]. All the reagents required for FDA staining was procured from Sigma Aldrich. A stock solution of 5 mg/ml FDA (Sigma-Aldrich, MO, USA) in acetone was prepared and stored at -20˚C. A fresh working solution of 20 µg/ml FDA was prepared daily by dissolving 10 µl of stock solution in 2.5 ml of 40% acetone in phosphate buffered saline at pH 6.8. Acid alcohol that is used in TB fluorescent microscopy was only used and 0.5% $KMNO_4$ was prepared in distilled water and used as counterstain. Smears made from the mucopurulent part of

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the sputum samples are to be air dried and are stained with FDAworking solution for 30 minutesfollowed by washing the slide with distilled water, and decolourization with 0.5% acid alcohol for 3 min and was counterstained with 0.5% $KMnO_4$. Slides after the staining was covered with 5% phenol for 10 minutes to render the smear non-infectious before examination[10]. Smears were examined by Zeiss (Heidenheim, Germany) iLED microscopes using the 100x objective with oil immersion without using microscopy cover slips.Any presence of greenish rods against dark background indicates the presence of viable bacilli **(Figure 1).**Presence of atleast one fluorescent bacillus per 100 high-power fields is considered as FDA positive[11]. The results of both the Auramine O staining and FDA staining technique are recorded and all smear positive specimens were processed for digestion and decontamination through standard sodium hydroxide-N-acetyl-L-cystein (NaOH-NALC) method and subjected to solid Culture. LJ Media was prepared in-house using standard precautions. Centrifuge deposits of the samples was inoculated on two Lowenstein-Jensen slants (L-J slants) and incubated at 37° C till growth appeared on the slants or till two months, whichever being earlier. After the culture growth obtained, the organisms were confirmed as MTBC using SD BIOLINE TB AG MPT 64Ag ICT Test[12].

3.Results:

A total of 142 smear positive sputum samples were included in the study, of which 94 (66.20%) were collected from Males and 48 (33.80%) were from Females. All the 142 smear positive sputum samples were subjected to Auramine O florescent staining, FDA staining and Culture on LJ-Media. Of 142 smear positive sputum samples, 45 (31.69%) were graded as scanty, 61 (42.95%) were 1+, 23 (16.19%) were 2+ and 13 (9.15%) were 3+ through florescent microscopy. **(Table 1)**. Simultaneously, the samples were processed for FDA staining for detection of viable acid fast bacilli. Of 142 samples, 114 (80.28%) detected the presence of Viable acid fast bacilli. The results of FDA positivity were confirmed by atleast two readers to avoid false positivity and negativity of the results. The results of FDA staining were then compared with the grading of Auramine O staining. 100% of the specimens having higher grade (2+ and 3+) were positive by FDA staining and 93.44% (57/61) of 1+ and 46.66% (21/45) of scanty smears were also positive by FDA staining. **(Table 2).** All the 142 specimens were digested and decontaminated and subjected to culture on LJ Media for isolation of MTB and MTB was isolated in 115 specimens of which 114 specimens were FDA positive. Results of Auramine O staining, FDA and culture were compared and are tabulated **(Table 3).** Considering culture as gold standard, FDA staining had a sensitivity of 99.14% and specificity of 96.43%. Positive predictive value, Negative predictive value of FDA staining was 99.14 and 96.43% respectively. Diagnostic accuracy of FDA staining in our study was found to be 98.61%.

4.Discussion:

National Tuberculosis Elimination Programme (NTEP) in India uses sputum smear microscopy as the basic diagnostic tool for Tuberculosis. The conventional ZN staining has been now replaced by fluorescent microscopy, which can detect 10% more TB cases and also has minimized the time taken to read ZN stained smear to 25% and also has its own benefits. The LED light source which is being used for fluorescence microscopy can be used to implement FDA vital staining method, which can be used to predict culture results and also can be used to monitor the treatment response. This vital staining technique has its own advantage of less

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turnaround time, which takes less than 1 hour to read the results and would only require the basic skills. Quantitative cultures would require long turnaround time. Other limitations such as false positive rates in newer techniques that utilizes DNA based amplification assays would require a sophisticated setting which cannot be possible in resource limited settings. Hence, there is a need for few more rapid and curate tests with low turnaround time and can be easily performed at resource limited settings. Hence, we tried to evaluate FDA vital staining technique in detecting viable acid fast bacilli and these results were compared to that of solid culture. FDA, a salt of fluorophore fluorescein has an ability to enter the viable Mycobacteria through hydrophobic cell membrane and get rapidly hydrolysed by cell esterases, this reaction takes place only in the viable cells and enables to fluoresce under LED fluorescent microscope and in contrast the dead bacilli lacks this activity and do not fluoresce[14,15]. Of 142 smear positive clinical specimens tested, 114 specimens were FDA positive indicating the presence of viable bacilli and 27 specimens were ZN positive but FDA staining and culture negative. This may be due to delay in smear conversion. Most of the peripheral laboratories relay on the results of smear microscopy and patients with delayed smear conversion will continue to take ATT until the availability of culture and DST results. Hence, introduction of FDA microscopy can improve the efficiency of smear microscopy services in resource limited settings and probably FDA staining can be considered as an alternative method for culture in resource limited settings.Our study had demonstrated sensitivity and specificity for FDA stain to be 99.14% and 96.43% respectively when considered culture as gold standard. Similar results have been reported by various other studies [11,15,16,17]. Designated Microscopy centres in India is already equipped with LED microscopes phased under NTEP. With high sensitivity and specificity for FDA staining, we recommend the use of FDA staining besides AFB microscopy. This would help in identifying treatment failure cases and help us to know the possible causes in delay in smear conversion.

The results of FDA staining when compared with culture results two had discordant results, of which one was graded 1+ through ZN staining, FDA negative, culture positive and the other was ZN negative, FDA positive and culture negative. The FDA negative and culture positive results may be because of spontaneous hydrolysis of FDA which can happen due to long term storage and also may be due to the poor esterase activity in some bacilli. FDA positive and culture negative results way be due to over decontamination of the specimens. Despite of high sensitivity and specificity, FDA staining has few minor limitations like the stock solution to be frozen at -20^0 c and working solution need to be prepared freshly whenever required. Smears have to be examined at the earliest as there is a chance of fading. Similarly advantages of this staining technique such as cost of reagents are similar to that of reagents being used in Fluorescent staining and hence can be used in peripheral laboratories having LED microscopy facility.

5.Conclusion:

Being a simple and economic method, FDA staining can be implemented for detecting the viable acid fast bacilli in sputum smear specimens and would be more valuable if done on follow-up specimens. Implementation of this method in peripheral laboratories with limited access to culture and drug susceptibility testing would help in minimizing the culture and drug susceptibility testing load in referral centres. But this needs further more evaluation by using this technique on followup specimens. FDA negative results during the follow up could be a surrogate marker for microbiological conversion. However, studies involving large number of samples including the followup specimens is very much needed to implement this technique as a valuable diagnostic tool for detecting viable acid fast bacilli under NTEP.

Conflicts Of Interest: No potential conflict of interest relevant to this article was reported.

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Table 1: Afb Smear Positive Grading Through Auramine O Staining:

Smear Grading	Positive	Percentage (%)
Scanty	45	31.70
1+	61	42.95
2+	23	16.20
3+	13	9.15%
Total	142	100%

Table 2: Comparison Of Fda Staining With Auramine Grading:

Auramine O smear grading	Positive	Percentage (%)	FDA positive	Percentage (%)
Scanty	45	31.70	21	46.66
1+	61	42.95	57	93.44

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2+	23	16.20	23	100
3+	13	9.15	13	100

Table 3: Comparison Of Fda Staining & Culture Results With Grading Of Zn Staining:

ZN grading	ZN Positive	Culture positive	Culture negative	FDA positive	FDA negative
Scanty	45	21	24	21	24
1+	61	58	03	57	04
2+	23	23	00	23	00
3+	13	13	00	13	00

Figure 1: Picture Showing Fda Positive Afb: