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Evaluation of A Method For Detection of Viable Mycobacterium tuberculosis Using LED Microscopy, In Sputum Specimens For Follow-Up of TB Treatment

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Review Article

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ABSTRACT

Following up the response of patients to the anti-Tuberculosis (TB) Treatment (ATT), requires basic and effective treatment monitoring tools. The commonly used monitoring tool used in peripheral TB laboratories in resource-limited settings is microscopic observation of Acid Fast Bacilli using Ziehl-Neelsen (ZN) or Auramine OFluorescent staining. If the patient is smear positive at the third month following treatment commencement, follow up for viability of the bacillus in the sputum, is done by Mycobacterium tuberculosis (M.tb) culture and drug susceptibility testing (C & DST).

Smear positivity at the fifth month or later following treatment commencement, would signal possible treatment failure. It is significant to note that's mear microscopy cannot distinguish between viable and dead bacilli. A large number of patients on treatment may continue to cough up dead bacilli from necrotic lung cavities, and hence be classified as "smear positive" though they respond to treatment. They are hence at risk of receiving prolonged or revised treatment regimens in settings with limited access to *M. tuberculosis* culture. Sputum culture can identify viable bacilli, but requires several weeks to arrive at the results, skilled laboratory staff and infrastructure.

Recent studies propose as imple method for TB treatment monitoring that can be performed at the peripheral TB laboratories with pre-existing infrastructure. It is a Point of Care (POC) technique based on the fluorescent viability marking capability of fluorescein-diacetate (FDA), as a stain for sputum smear microscopy.

The effectively of fluorescein-diacetate (FDA) staining in the detection of viable *Mycobacterium tuberculosis* in 567 sputum samples from follow up PTB cases was compared to growth on automated liquid culture systems (97.64% culture positivity). FDA sensitivity was moderate (97.62%, with a Confidence Interval of 95%), and specificity was (98.4%). The negative predictive value of 94.8% was encourage in ganda negative likelihood ratio of 0.2 suggested use of this method for ruling out treatment failure in low income and high burden settings.

INTRODUCTION

Checking the prognosis of PTB patients on Anti-TB Treatment (ATT) is important for early detection of treatment failure or drug resistance [1]. The monitoring tool commonly used in resource-limited and high burden settings is usually microscopic observation of sputum smears using Ziehl-Neelsen (ZN) or Auramine Ostaining [1,2]. A positive smear in the third month following commencement

of ATT or later is further investigated by C&DST for M. tb. Smear positivity in the fifth month or later would mean poor response or failure of the treatment [1].

It is significant to note here that smear microscopy using ZN or Auramine O staining, cannot differentiate between viable and dead bacilli. A large number of patients on ATT continue to cough up dead bacilli from necrotic lung cavities and remain smear positive though they respond to treatment [1,3-7]. These patients are at risk of receiving prolonged or new treatment regimens unnecessarily.

Mycobacterial culture is the only method that can conclusively identify viable bacilli. Unfortunately it takes many weeks for the results to be read and also requires highly skilled human resources apart from expensive laboratory infrastructure. Contemporary studies propose as imple and on the spot method for ATT monitoring, based on staining the sputum smear from follow up cases using fluorescein-diacetate (FDA), afluorescent viability marker, prior to microscopy [8-10].

METHODS

The FDA vital staining method was compared to M.tb culture using 567 sputum samples of follow up PTB patients, received at the Intermediate Reference Laboratory (IRL) at SDS Sanatorium, NIMHANS, Bangalore, during routine ATT monitoring during the second, third (if positive during the second month), fifth, and sixth month for treatment of new PTB cases and during the third, fourth (if positive during the third month), fifth and the eighth month for the treatment of previously treated PTB cases.

Two consecutive specimens per follow up month for every case were examined microscopically using ZN staining [2]. Smear positive specimens by ZN staining alone were included for the study. They were stained using FDA for smear microscopy within 2 days of sample receipt [9]. FDA stock solution (Fluorescein Diacetate - CAS 596-09-8 –Calbiochem Sigma Aldrich), 25mg/ml in acetone, stored at —20°C) was used to prepare fresh staining so- lution (0.5mg/ml) in phosphate-buffered saline (pH 7.3, with 0.05% Tween 80). After air drying, FDA smears were examined by fluorescence microscopy at ×1,000 magnification using a Labomed Fluorescence LED Microscope LX 400 EFL, equipped with a FluoLed Blue (480 nm) light-emitting diode (LED) and a 48-75 mm band-pass filter. An FDA smear was defined as positive (FDA+) when at least 1 fluorescent bacillus/100 high-power fields was observed [2].

The remaining sample was used to set up culture for *M. tuberculosis* on solid and liquid media at the Intermediate Reference Laboratory (IRL) at SDS Sanatorium, NIMHANS, and Bangalore. Decontamination of the sample was done as per the Standard Operating Procedures (SOPs) prescribed by the National Tuberculosis Elimination Programme (NTEP), using N-acetyl-L-cysteine-sodium hydroxide (NaLC – NaOH), with a final NaOH concentration of 2%for 15 min. One culture tube for incubation in the automated MGIT 960 liquid culture system and two tubes with the solid egg-based Lowenstein-Jensen culture medium were inoculated per specimen.

Smears from positive cultures were observed microscopically following FDA staining, ZN staining, and identification of the culture as M.tb was done using the rapid The lateral flow immune chromatography test (Capilia TB test) has also been reported for differentiation of the *M. tuberculosis* complex from NTM. If viable mycobacteria are present in an inoculated specimen, they will grow in the MGIT medium and will be detected visually as well as by fluorescence. Report results only when a MGIT tube is positive by the instrument and smear made from the positive broth is also positive for FDA, AFB. 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 (Capilia TB test) for all three culture tubes inoculated on solid and liquid culture was positive. Contaminated cultures were excluded from the performance analysis.

567 Ziehl-Neelsen stained smear positive samples (ZN+) from follow-up cases were included between June 2019 and September 2019. Of them, 79.4% were scanty ZN positive, defined as slides with 1 to 9 bacilli/100 high power fields and 67.2% were at the end of the intensive phase or the prolonged intensive phase of their treatment regimens but the culture positivity is only 80 (14.10%).

RESULTS AND DISCUSSION

Among the total 567 specimens, 250 were FDA Smear positive 317 were indicated to be FDA negative among the 250 FDA smear positive, 244 (97.62%) were found to be positive by *M. tuberculosis* culture, 2 (0.80%) were NTM positive, 2 (0.8%) were culture negative, and 2 (0.8%) was contaminated. In all, 250 (26.4%) of the follow up samples were FDA smear positive, of which 175 (70%) of these were found to be scanty positive. The sensitivity of FDA smear was 97.6 %, and its specificity was 98.4 %.

False positive results among the FDA stained slides were not all see in the FDA (246/250, 98.40%) when compared with ZN positive smear 80/567 (14.10%) to culture (**Tables 1-3, Figures 1 and 2**).

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Table 1. Numbers of included ZN+ sputum specimens and FDA Sputum +ve specimens. ZN+, Ziehl-Neelsen, FDA stain-positive sputum smear; MTB+, *Mycobacterium tuberculosis*-positive culture; NTM+, non tuberculous mycobacterium-positive culture in Different phases of treatment; ZN scanty+, 1 to 9 bacilli/100 HPF; ZN > 1+, >10 bacilli/100 HPF, FDA Scanty +ve1 to 4 bacilli/40 HPF; FDA> 1+, >10 bacilli/40 HPF, (ZN Smear Sampling).

	Total Sample	ZN +ve Smear	FDA +ve Smear	Culture Cont	End of Intensive phase ZN +ve	End of Intensive phase FDA +ve	End of Treatment ZN +ve	End of Treatment FDA +ve
	567	567	250					
Culture +ve		90	236	10	80	187	2	15
Culture-ve		477	2		330		35	
NTM						2		

Table 2. This represents the total positive MTB Growth, No Growth, NTM, and Contamination in different phase of sampling.

	End of Intensive Phase	Prolonged Intensive Phase	Continuation phase	End of Treatment
MTB Growth	80	5	3	2
No Growth	330	65	35	35
NTM + ve	0	0	2	0
Contamination	9	0	0	1

Figure 1. Numbers of included ZN+ sputum specimens with specimen characteristics. ZN+, Ziehl-Neelsen stain-positive sputum smear; MTB+, *Mycobacterium tuberculosis*-positive culture; NTM+, non tuberculous mycobacterium-positive culture; ZN scanty+, 1 to 9 bacilli/100 HPF; ZN > 1+, >10 bacilli/100 HPF (FDA Smear Sampling).

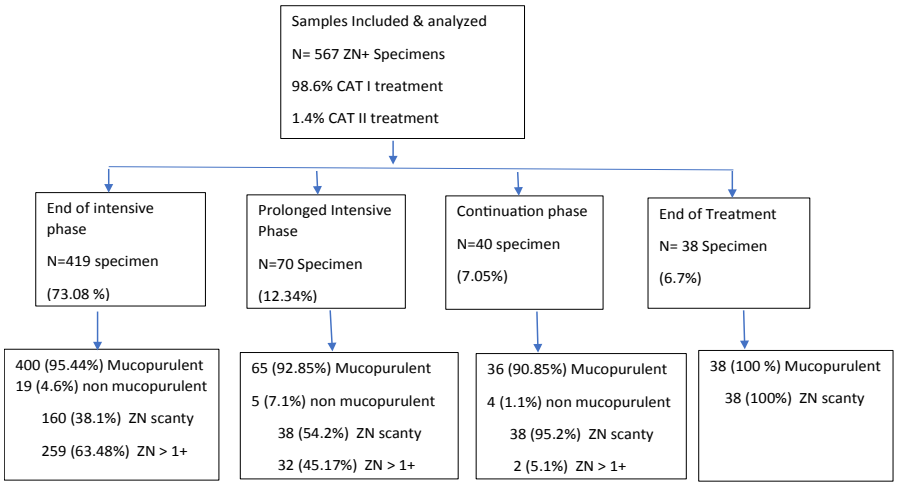

Figure 2. Numbers of included FDA+ sputum specimens with specimen characteristics. FDA+, Fluorescein Di Acetate stain-positive sputum smear; MTB+, *Mycobacterium tuberculosis*-positive culture; NTM+, non tuberculous mycobacterium-positive culture; FDA scanty+, 1 to 9 bacilli/100 HPF; ZN > 1+, >10 bacilli/100 HPF.

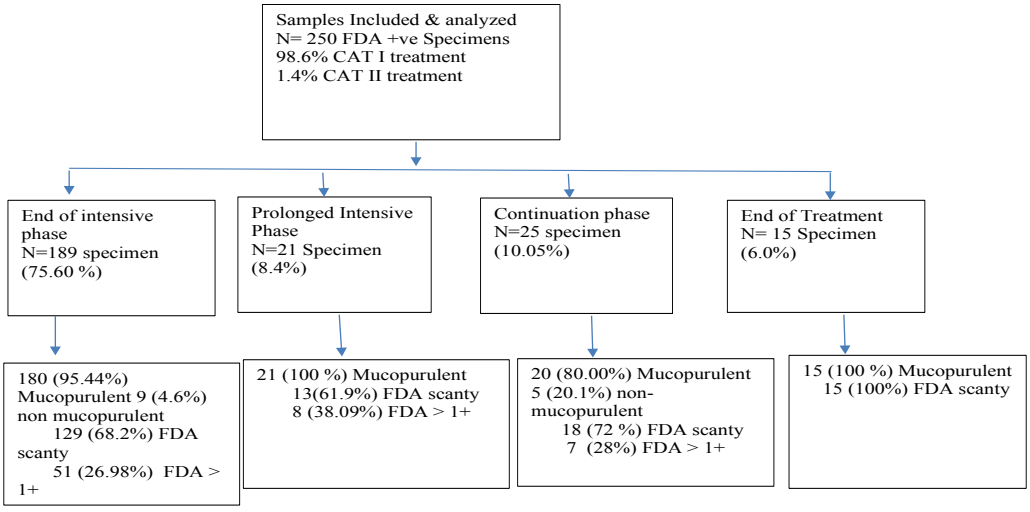

Table 3. This represents the total positive MTB Growth, No Growth, NTM, Contamination in different phase of sampling (FDA Smear).

	End of Intensive Phase	Prolonged Intensive Phase	Continuation phase	End of Treatment
MTB Growth	187	19	23	15
No Growth	0	0	2	0
NTM + ve	2	0	0	0
Contamination	0	2	0	0

DISCUSSION

The overall performance of FDA was good. The study population consisted mostly of scanty ZN-positive specimens from patients showing delayed response to treatment, accompanied by a low culture positivity rate. Indeed, the FDA accuracy seemed to be perfect specific to that of specimen and their culture. Furthermore, FDA specificity may have been understood by the culture-negative results. With a very low culture contamination rate, it is likely that some bacilli, probably from pauci bacillary specimens, could have been killed during the process of decontamination [11]. Furthermore, Schramm et al. observed that the viability of bacilli as defined by FDA positivity (Fluoro chrome activation by enzymatic activity) need not necessarily signify "viability" of the bacillus which is defined by culture as the ability to multiply [12]. This could be more obvious in specimens from patients in the early treatment phases.

In this study to assess the viable staining of bacilli using the FDA staining method, the performance was accurate enough to propose the FDA smear method as a standalone tool for TB treatment monitoring. In resource limited settings, this method maybe found to be valuable to identify cases requiring culture. Such application needs further evaluation among populations with a higher proportion of treatment failures.

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