Original article:

Use of fluorescein diacetate (FDA) staining to detect viable *Mycobacterium tuberculosis*

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Abstract

Objective: Drug resistant tuberculosis has long been a common problem prevailing in developing countries including Bangladesh. Present study focused on the rapid identification of live *Mycobacterium tuberculosis* among treatment failure cases. ***Materials and Methods***: Sputum samples from a total of 100 category-I and category-II treatment failure cases, assumed as multidrug resistant tuberculosis, were studied through fluorescein diacetate (FDA) staining under light emitting diode (LED) fluorescence microscope. Considering culture method as gold standard, we also compared the results of FDA staining with that of auramine O staining. ***Results****:* A total of 85% acid-fast bacilli were detected by FDA staining, 82% by auramine O staining and a total of 85% isolates were detected in Lowenstein-Jensen (LJ) culture. The sensitivity of FDA staining (96.47%) was estimated to be slightly higher than that of auramine O staining (91.76%). Moreover, 76.47% cases were detected as multidrug resistant tuberculosis (MDR-TB). ***Conclusion***: Taken together, FDA staining method has been proposed to be appropriate for the rapid diagnosis of drug resistant tuberculosis.

Key words: Tuberculosis, fluorescein diacetate staining, auramine O staining, Lowenstein-Jensen culture, multi-drug resistance.

Introduction

Tuberculosis (TB), caused by the bacterium *Mycobacterium tuberculosis* (MTB) [1] affects lungs and other organs in the human body[2,3]. Approximately one third of the world's population is infected with *M. tuberculosis* leading to 10 million new cases each year [4,5]. Bangladesh ranks sixth among the highest TB burdened countries in the world with around 354,000 new cases every year and approximately 71,000 deaths annually[6]. Such a deadly situation is even worsening in many parts of the world, primarily because of the growing prevalence of multidrug resistance against tubercle bacilli[7].

In Bangladesh, National TB Control Program (NTP) began implementing Directly Observed Treatment Short Course (DOTS) to control TB and minimized the rate of multidrug resistant TB in 1993[6]. However, the success of tuberculosis control programs depends not only on successful completion of treatment but also on early diagnosis, steady monitoring, and response to treatment[8]. Category (CAT)-I includes new smear positive cases of pulmonary TB. Whereas, Category-II pulmonary TB includes those patients who had failed previous TB treatment, relapsed after treatment, or defaulted during previous treatment. Since such patients have already been exposed to anti-tuberculosis agents, they are at high risk for harbouring multi-drug resistant strains[9]. Thus, the urgency in the accurate diagnosis of the disease and the necessity of quick detection of *M. tuberculosis* from the speculated treatment failure TB cases led us to study the conventional methods as well as to introduce the FDA staining as a new reliable and quick method of TB diagnosis in Bangladesh.

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Diagnosis of *M. tuberculosis* in Bangladesh is currently based mainly on acid-fast staining and culture of decontaminated samples on solid media or radiometric liquid culture (BACTEC technique) [10,11]. *M. tuberculosis* is grown on a selective medium known Lowenstein-Jensen (LJ), Kirchner, or Middlebrook media (7H9, 7H10, and 7H11) where only 10 to 100 organisms are sufficient for detection [12]. The sensitivity of culture method is 80-93% and the specificity is 98% [12]. However, the slow growth of most pathogenic mycobacteria results in delays in definitive diagnosis by culture method [13]. Another diagnosis is based on the the auramine O staining, a histological technique used to visualize acid-fast bacilli using fluorescence microscope [14]. Although the auramine O staining is not specific for the acid-fast tubercle bacilli like the Ziehl-Neelsen staining, it is more affordable and more sensitive and therefore is often utilized as a screening tool [14]. However, viable and non viable acid fast bacilli cannot be distinguished by either Ziehl–Neelsen staining or auramine O staining. Such a differentiation is emergently asked to address the treatment failure cases, mostly MDR-TB. Another approach for rapid detection is based on the polymerase chain reactions (PCR) of the suspected virulence gene(s) [15]. However, due to the requirement for high logistics, this method seems inconvenient in our country.

Overall, due to limitations of the currently used diagnostic methods stated above, we propose the use of FDA staining which has been highly predictive of mycobacterial growth in culture, and for the diagnosis of true treatment failure cases [16,17]. Fluorescein diacetate, a salt of the green fluorophore fluorescein, is detectable by fluorescence microscopy only after its cleavage by cell esterases. These are only supposed to be present in viable cells [18]. Based on these facts, the present study attempted to perform FDA staining to detect viable *M. tuberculosis* from CAT-I and CAT-II failure patients. Our study also undertook the detection of *M. tuberculosis* by auramine O staining and conventional culture method, and then we compared the results obtained from FDA staining and auramine O staining methods with that of the conventional culture method to determine the sensitivity and specificity of the methods.

Another aspect of our current study focused on the multidrug-resistant tuberculosis (MDR-TB), which has recently emerged as a global health threat [19]. The incidence of MDR among *M. tuberculosis* isolates, as well as the arrival of HIV/AIDS resulted in an increase in outbreaks of MDR-TB [19-21]. In the early 1990s, drug resistance surveillance was resumed in developed countries, but the true incidence remained unclear [21]. Therefore, mycobacterial cultures and susceptibility testing must be conducted for the eradication of tuberculosis. Thus, we performed the conventional drug susceptibility test (DST) against the first line anti-tubercular drugs to detect the frequency of MDR-TB, to which FDA staining under LED fluorescence microscope was further linked as the diagnostic method.

Materials and methods

The study was carried out at National Tuberculosis Reference Laboratory (NTRL) of National Institute of Diseases of the Chest and Hospital (NIDCH), Bangladesh, from July 2010 to March 2011. 100 patients of all ages and both sexes, who had already received the first line anti-tubercular drugs (isoniazid, rifampicin, streptomycin and ethambutol) were enrolled in the study to detect the treatment failure cases and the prevalence of MDR-TB.

Sputum collection, Smear preparation and Microscopic Observation

One spot morning sputum sample was collected in a sterile container. Smear was prepared from yellow purulent portion of the sputum using a sterile bamboo stick. The smear was spread evenly, air-dried for 15 minutes, and fixed by placing the slide over a hot plate at 85 ^{O}C for 3 minutes. Two direct smears were prepared for staining by FDA and auramine O staining techniques, respectively, and were examined under LED fluorescence microscope (Primostar, Carl Zeiss LED, Germany) at 455

Fluorescien Diacetate (FDA) Staining

The air dried, non fixed smears were placed in a Petri dish on staining rods, covered with a piece of filter paper soaked with the FDA working solution, and incubated at 37 ^{O}C. After 30 minutes, filter paper was removed and 0.5% acid alcohol was applied for 3 minutes, followed by the addition of 5% phenol for 10 minutes. Smears were removed from Petri dish, dipped briefly in water and were kept in a dark place [18,22]. Finally, the dried smear was

examined under LED fluorescence microscope at 455 nm (400× magnifications).

Auramine-O staining

Smear was covered with 0.1% auramine solution for 15 minutes, decolorized with 0.5% acid-alcohol for 3 minutes, and flooded by 0.3% methylene blue for 1 minute[11,23]. After drying for 30 minutes, the smear was examined under LED fluorescence microscope at 455 nm (400× magnifications).

Culture method for isolation and identification of M. tuberculosis

All samples included in this study were decontaminated by modified Petroff method using 4% NaOH. Briefly, the sample (2-5 ml) was decontaminated by mixing properly with the equal volume of 4% NaOH, and was allowed to stand for 15 minutes. 7mM phosphate buffer saline (PBS) solution (pH 6.8) was added up to 45 ml mark of the tube to neutralize the effect of 4% NaOH. The sample was then centrifuged at 3000g for 15 minutes, and the pellet was subjected for inoculation into L-J medium to isolate *M. tuberculosis*. In this study, 3 or 4 drops of decontaminated and concentrated sample was inoculated on two slopes of LJ culture media. The media was then incubated at 37 ^{O}C after inoculation[11]. The final species identification of *M. tuberculosis* was based on the characteristics such as slow growth up to 45 days, appearance of small and yellow or buff colored colony, and the typical biochemical traits. *P*-nitrobenzoic acid (PNB) sensitivity test was also done since PNB is a selective inhibitor of *M. tuberculosis* [20]. L-J medium containing PNB was inoculated with 2 drops of bacterial suspension and was observed within 11 days for the presence or absence of growth. *M. tuberculosis* H37Rv, the reference strain of *M. tuberculosis,* was used as positive control for the identification

Drug susceptibility test (DST) by proportion method

One loopful colony of *M. tuberculosis* was picked and the loop was gently shaken over 5-7 sterile glass beads in a tube. After 30 minutes, 2 ml of tween 80 solution was added to the homogenous upper part of the supernatant with similar dimension of the 0.5% Mac Farland standard[11]. Serial dilutions of the bacterial suspension were prepared with normal saline up to 10^{-5}. L-J media containing the drug isoniazid (INH), rifampicin (RM), streptomycin (SM), ethambutol (EMB) and the media free of any drug (control) were inoculated with the inoculum (from the dilutions 10^{-3} and 10^{-5}).

Determination of sensitivity, specificity, positive predictive value, negative predictive value and accuracy

All data from this study were statistically analyzed for the comparison of different detection methods and to measure the sensitivity, specificity, positive and negative predictive values of the microscopic methods. Sensitivity and specificity are terms used to describe the value of tests. These values can be determined on the basis of true positives, false positives, true negatives, and false negatives according to equations given by Kausar (2008)[24]. This study was appoved by 'Research and ethics committee of National Tuberculosis Reference Laboratory (NTRL) of National Institute of Diseases of the Chest and Hospital (NIDCH), Bangladesh'.

Results

Among 100 TB cases, 30 were found within the age group of 15-25 years. 10 cases were detected in the age group of 55 years or more. A total of 10 patients were found CAT-I failure- and 90 patients were detected as CAT-II failure cases. Among the patients, 86% were illiterate and 14% were found to be educated. When the occupation of the patients was considered, 40% and 30% of the patients were labour and garment workers, respectively, and others were service holders (15%), housewives (10%) and students (5%).

Detection of M. tuberculosis

For the detection of *M. tuberculosis* from treatment failure TB cases, we performed FDA staining, auramine O staining and culture methods. In this study, under LED fluorescence microscope (after FDA and auramine O staining), acid fast bacilli (AFB) were found which appeared as typical rods with some curved or bent, bright yellow or greenish against a black background (figure IB). The sample observed under microscope gave results on the basis of the quantity of bacilli. At 400x magnification, more than 50 bacilli per microscopic field was considered three plus (3+) results whereas between 5 to 50 bacilli/field considered as two plus (2+) results. One plus (1+) and scanty results were determined by visualizing 20-299 bacilli/1 length and 1-19 bacilli/1 length of microscopic fields, respectively. 85% and

82% positive cases were detected as acid fast bacilli under LED fluorescence microscope by FDA staining and auramine O stainimg methods, respectively. 85% samples were found to be culture positive.

The isolates were identified as *M. tuberculosis* by different biochemical procedures and PNB sensitivity tests. All the isolates showed positive reaction in nitrate reduction test (figure IIA) which is a typical characteristics of *M. tuberculosis*. In catalase test, the isolates showed catalase positive reactions and were identified as *M. tyuberculosis*. Furthermore, absence of mycobacterial growth on L-J media containing PNB (500 ?g/ml) indicated that the isolates were sensitive to PNB which is another feature of *M. tyuberculosis* (figure IIC). After identification of the isolates as *M. tuberculosis*, the results obtained from FDA staining and auramine O staining were compared to that of culture methods for statistical validation and then the drug susceptibility of the identified isolates were examined.

Comparison of results to statistically measure the sensitivity and specificity of the methods

In order to find out which microscopic method was more sensitive and accurate for the rapid detection of viable *M. tuberculosis* from the treatment failure TB patients, the results of FDA staining and auramine O staining were compared with that of the culture method to measure the sensitivity, specificity, positive and negative predictive values and accuracy of different methods (tables I & II). Table I shows the true positive , true negative , false positive and false negative results in cases by FDA staining. From these results, we found that the sensitivity of FDA staining was 96.47% and specificity was 80%. The positive and negative predictive values were 96.47% and 80%, respectively, and the accuracy was detected as 94%.

The sensitivity of auramine O staining was 91.76% and specificity was 73.33% compared to L-J culture method. The positive and negative predictive values were 95.12% and 61.11 %, respectively, and the accuracy was 89%. The results revealed that the FDA staining was more sensitive, specific and accurate method than the auramine O staining to detect *M. tuberculosis*.

Frequency of multidrug resistant isolates

Next we turned to study the prevalence of multidrug resistant tuberculosis among the patients who have been treated with anti-tubercular drugs. Out of 100 TB treatment failure cases, 85 isolates, identified as *M. tuberculosis*, were tested for their resistance against isoniazid, streptomycin, rifampicin and ethambutol for appropriate treatment of TB patients. According to our study, 76 (89.41%) and 70 (82.35%) isolates showed resistance to rifampicin and isoniazid, respectively. The frequency of resistance against ethambutol and streptomycin was 55 (64.70%). The resistance was found higher against rifampicin and isoniazid. As these two drugs are the most commonly used first-line drugs for the treatment of tuberculosis, higher resistance against these drugs indicated the possibility of treatment failure. The frequency of multidrug resistance was then determined to measure the prevalence of MDR-TB. Table III shows resistance against multi drugs. The highest rate (47.05%) was observed against the four first line drug-combination and the lowest (5.88%) was observed against the combination of ethambutol, isoniazid and rifampicin. Overall, 76.47% isolates were found to be multidrug resistant and 8.23% were found sensitive to all drugs. The results revealed that the rate of MDR-TB was higher among the treatment failure cases.

Discussion

The problem of TB infection has been aggravated by the increasing density of population, rapid uncontrolled urbanization, overcrowding, poverty, malnutrition and illiteracy, which have long been prevailing in Bangladesh[25,26]. Moreover MDR-TB is increasing day by day and coinfection with HIV is also contributing to the growing burden of tuberculosis [27]. Early case detection can be done by sputum examination for acid fast bacilli from suspected cases of pulmonary tuberculosis [28]. However, drug resistance interferes the effective treatment of tuberculosis with first line anti tubercular drugs [28]. Thus, in absence of a truly effective tuberculosis vaccine, treatment of active cases remains the most important component of tuberculosis control programs. For the purpose of such treatment activities to be efficient and effective, rapid and accurate diagnosis is essential [29].

In this study, we suggest a more rapid and accurate diagnosis of drug resistant-TB by using FDA staining. The study sorted out the prevalence of MDR-TB among those who have been previously infected as

well as have been medicated with TB suppressive drugs. Our study also addressed different diagnostic methods to compare which method was more reliable and more accurate. In Bangladesh, the prevalence of TB is higher which led the Government to initiate Directly Observed Treatment Short Course (DOTS) strategy to ensure a proper follow up to detect the treatment failure cases among TB infected patients. However, proper diagnosis of MDR-TB requires differentiating between live and dead bacilli. Considering this factor, FDA method is more preferable than the other methods for its capability of rapid detection of live bacilli [18,30].

MDR-TB has increased in incidence and interferes with TB control programs, particularly in developing countries, where prevalence rates are as high as 48% [31]. Detection of drug-resistance *M. tuberculosis*is of primary importance for both patient management and infection control [32]. Considering these, we screened the presence of multidrug resistant tuberculosis among patients who were already under treatment and observed the present situation of MDR-TB in Bangladesh by testing the isolates' resistance or sensitivity against streptomycin, isoniazid, rifampicin and ethambutol. Our results revealed that the frequency of drug resistance was relatively higher for the treatment failure cases. We found 76.47% cases as MDR-TB which is alarming and thus possesses significant threat to public health.

In fine, our study clearly indicates a significant high rate of drug resistance among TB patients in Bangladesh. We propose that FDA staining using fresh sputum can be used for early and accurate diagnosis of treatment failure TB cases as the method is capable to distinguish live tubercle bacilli from the dead ones. The FDA method is also more sensitive, specific and accurate method than the auramine O staining. Thus the present study might have a significant contribution to control and eradicate tuberculosis completely from Bangladesh.

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Conflict of interest

Authors have no potential conflict of interests.

Table I. Comparison of results of FDA staining with Culture

FDA staining	Culture results		
	Positive	Negative	Total
Positive	82 (96.47%)	3 (20%)	85 (85%)
Negative	3 (3.52%)	12 (80%)	15 (15%)
Total	85	15	100

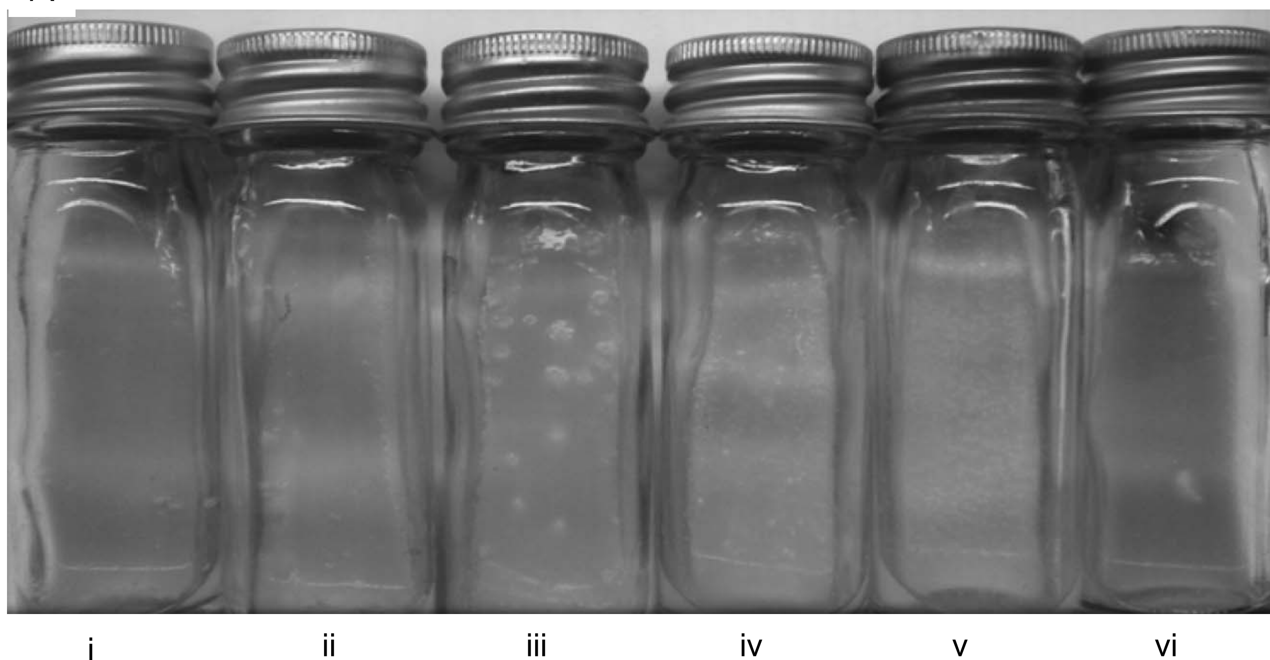
Table II. Comparison of results of auramine O staining with Culture to Detect *M. tuberculosis*

Auramine O staining	Culture results		
	Positive	Negative	Total
Positive	78 (91.76%)	4 (26.66%)	82 (82%)
Negative	7 (8.23%)	11 (73.33%)	18 (18%)
Total	85	15	100

Table III. Resistance to multi drugs

Drugs used	Number of resistant isolates
H + R	8 (29.41%)
H + R + E	5 (5.88%)
H + R + S	12 (14.11%)
H + R +E + S	40 (47.05%)

H: Isoniazid (2 µg/ml) R: Rifampicin (40 µg/ml)
E: Ethambutol (2 µg/ml) S: Streptmycin (4 µg/ml)



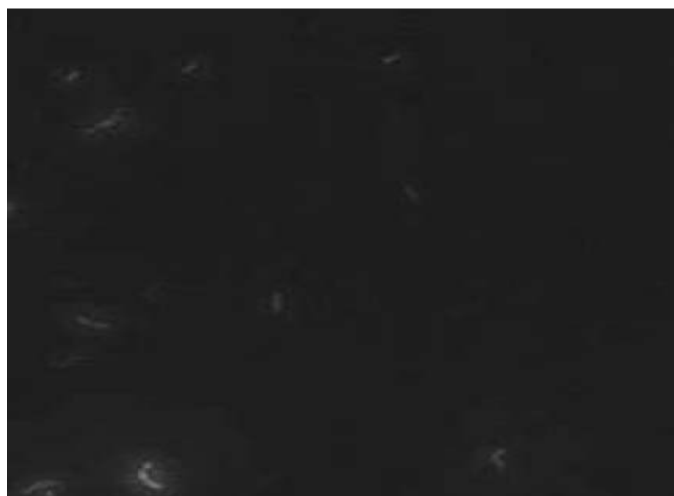


Figure I. *Appearance of Mycobacterium tuberculosis on L-J media and under LED fluorescence microscope. A. Large, yellow colonies of Mycobacterium tuberculosis were observed on L-J media. i. negative (no growth), ii. scanty (few growth), iii. + (1-90 colonies), iv. ++ (100 discrete colonies), v. +++ (confluent growth) and vi. contamination; B. Greenish, curved rod acid fast bacilli (AFB) were visualized under LED fluorescence microscope at 455nm after FDA staining (400x magnifications).*

A. i ii iii iv

B. vi v iv iii ii i

i ii iii

A.

Figure II. Biochemical identifications and PNB sensitivity test. A. Red coloration indicates that the isolate-ses were nitrate positive. i. positive control, ii, iii & iv. Positive reactions; B. Oxygen bubbles were formed in the unheated tubes. i. positive control (M. tuberculosis H37Rv), ii, iii, iv, v & vi. Positive reactions; C. No growth on L-J media containing PNB in the PNB sensitivity test. i. positive control (M. tuberculosis H37Rv), ii & iii. PNB sensitive.

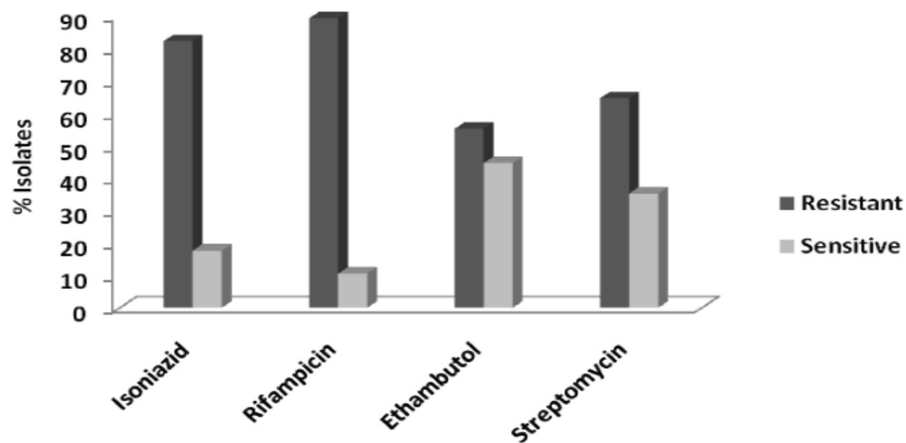


Figure III. *Frequency of drug resistance of the isolates. The isolates showed relatively higher rates of resistance against rifampicin (89.41%) and isoniazid (82.35%). 64.70% and 55.29% isolates were resistant against streptomycin and ethambutol, respectively.*

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