



Original Research Article

Predictive utility of fluorescent microscopy for detection of drug resistance in patient of pulmonary tuberculosis

Sushmita Mukherjee¹, Navinchandra M Kaore^{1,*}, Shilpa N Kaore², Surender Kaur³

¹Dept. of Microbiology, Raipur Institute of Medical Sciences, Raipur, Chhattisgarh, India

²Dept. of Pharmacology, All India Institute of Medical Sciences (AIIMS), Bhopal, Madhya Pradesh, India

³Dept. of Microbiology, Government Medical College, Rajnandgaon, Chhattisgarh, India



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ABSTRACT

Tuberculosis (TB) is a global health problem with 480000 new cases being reported annually, 9 percent of which exhibit extensive drug resistant TB. With 9.6 cases per lac population early detection of drug resistance at peripheral levels is must.

The study was planned to use Fluorescent microscopy (FM) with Fluorescein Diacetate Ethidium Bromide (FDA/EB) stain to evaluate the viability of the mycobacteria to predict the drug resistance.

This cross-sectional analytical study was carried out on all sputum smear positive patients after obtaining the Institutional Ethics Committee clearance over a period of 3 months. Samples were obtained on day 0, 3, 7 and 14. After staining with the working solution, observation was made by two independent observers for viable versus non-viable mycobacteria. FDA will stain the live bacilli and fluoresce green whereas dead bacilli lack the esterase activity and are counterstained by ethidium bromide and appear red. Data was maintained in MS Excel and analysed using tests of proportion and significance.

A total of 30 participants were included based on the inclusion criteria. There was a loss of follow up from 14 patients. 4/16 (25%) patients were found to harbor drug resistant mycobacteria strains based on decreasing ratio of viable to non-viable bacteria on followup. Results when compared with gold standard of Line Probe Assay (LiPA) was found to be highly significant with Chi square value of 11.73 and p value <0.001

This study establishes the utility of fluorescent microscopy using FDA and EB to detect drug resistance in patients of pulmonary tuberculosis by evaluation of viable and non-viable at peripheral microscopic centers
Key Message: Fluorescent Microscopy with FDA/EB staining holds a potential to predict drug resistance in sputum smear positive patients at peripheral designated microscopic centers which will reduce financial as well as human resource burden.

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1. Introduction

Tuberculosis (TB) has existed for centuries and still remains the global health problem of the 21st century. Globally about ten million people are infected with TB. In India, incidence of TB is approximately 28,00,000 which accounts for almost quarter of world's TB cases.¹

The Multi Drug Resistant TB (MDR-TB) cases, which means the patient is resistant to two most important 1st

line drugs, that is Rifampin and Isoniazid has been rapidly increasing along these years. Annually, around 4,80,000 new cases of MDR-TB have been recorded, out of which about 9% exhibit extensive drug resistance TB.² Early detection of the drug resistance is the need of the hour. According to the Global TB Report 2019, the number of MDR TB in India is 9.6 per lakh population.³

Conventionally, when a TB case is suspected, it is first of all subjected to sputum microscopy and examined. If found to be sputum positive, those patients are put under CAT I and are started with the treatment under intensive

* Corresponding author.

E-mail address: nkaore@gmail.com (N. M. Kaore).

phase for a period of 2 months. After two months, again the patient is examined for sputum positivity. If still the sputum microscopy is positive, then the patient is put under CAT II and are assumed to be drug resistant that is having MDR TB. Recently under Revised National Tuberculosis Control Program (RNTCP), now National Tuberculosis Elimination Program (NLEP) all the sputum smear positive samples are sent for detection of MDR status right on the first day by Line Probe Assay (LiPA) at Intermediate Reference Laboratories (IRL) as around 25-30% newly diagnosed cases are MDR TB cases. It is also important in view that globally only 5% cases of PTB are tested for drug susceptibility tests (DST).⁴

Fluorescent microscopy (FM) using the Fluorescein Diacetate Ethidium Bromide (FDA/EB) on the other hand, evaluates the viability of the bacteria. It uses the fatty acid ester fluorescein diacetate to measure the esterase activity of the bacilli. Esterase activity is only shown by the live bacilli and hence they fluoresce green. Dead bacilli lack the esterase activity and so do not fluoresce. They are counterstained by ethidium bromide and appear red.^{2,3,5,6}

In the intensive phase the viability of the mycobacteria will go on decreasing and non-viable bacteria will increase if the mycobacterium tuberculosis (MTB) are sensitive to primary drugs and vice versa.

Thus, this study was planned primarily to establish the utility of FM for the detection of drug resistance in patient of pulmonary TB by assessing the viability of MTB and compare the results with gold standard LiPA, so as to reduce the burden of sample examination by the health care workers at the IRL This would also be cost effective as LiPA at private setting is expensive and would lower the heavy sample load at the government setting.

2. Materials and Methods

This cross sectional prospective analytical study was carried out in the Mycobacteriology laboratory of the Department of Microbiology, attached to a Medical Institute in Central India over a period of 3 months for due approval from Research Advisory Committee (RAC) and Institutional Ethics Committee (IEC). The IEC was granted wide approval letter no. RIMS/ADMIN/261-C/2019 dated 11.07.2019.

Samples from all sputum smear positive patients diagnosed by bright field microscopy at the DMC and were willing for follow up were enrolled in the study whereas all repetitive samples and sputum smear negative samples as well as patients not willing to come for follow up were excluded from the study.

Initially all the sputum samples submitted for examination at DMC were processed. All the sputum smear positive patients were then enrolled for the study after written informed consent. The participants were followed up and mucopurulent sputum samples were

collected on Day 0, 3, 7 & 14 as per RNTCP protocol for assessment of viability by FM and examined immediately. All the patients who missed the follow up were later excluded from the statistical analysis.

2.1. Preparation of Stock Solution^{6,7}

2.1.1. Stock Solution

A stock solution of FDA was prepared to give a concentration of 5 mg/ml in acetone. 1 ml was distributed in aliquots and stored at minus 20°C. The stock solution of EB was prepared to give concentration of 2 mg/ml in 1/75 phosphate buffer saline (PBS) at pH 6.5 with 0.05% Tween 80.

2.1.2. Working Solution

The working solution was prepared immediately before staining. The FDA stock solution was diluted 1:5 in acetone and a 0.5 ml volume was then added to 4.5 ml volume of PBS-Tween 80.

The Stock solution of EB was diluted in 1: 250 in PBS-Tween 80 containing FDA as prepared above. The final concentration of FDA and EB in working solution was kept at 100 µg/ml and 8 µg/ml respectively.

2.1.3. Staining of smears

The smears prepared from the Day 0, 3, 7 & 14 were stained with working solution of FDA-EB. The prepared smears were covered with 0.5 ml of the working solution directly and covered with the coverslips and sealed with colorless nail polish. The slides thus prepared were incubated at 37°C for 60 minutes and then observed.^{5,6}

2.1.4. FM Observation

Stained preparation were observed using LED FM at 1000X with blue light at 480 nm. The viable mycobacteria were observed as bright green to yellow bacilli whereas non-viable bacilli will be observed as red. (Figures 1 and 2) The observation was made by two independent observers to remove the observer's bias. First 100 bacilli or less number if bacillary load is less were observed and viability and non-viability was observed and counted. The average of two independent observer was taken into account. The persistence of more number of viable mycobacteria against non-viable mycobacteria was considered as persistence of infection indicative of resistant strain causing PTB and vice versa.

After sputum positivity, when the sample was examined through FM and the ratio of Viable to Non-viable was recorded on 0, 3, 7 and 14th day and found to be in the decreasing ratio, then there is response to treatment. If not, that implies that treatment initiated for TB not effective and it is a case of drug resistant TB.

All the sputum smear samples detected on day 0 and put on anti TB treatment were sent for line probe Assay (LiPA)

as per RNTCP protocol for detection of the MDR-TB. The results of the LiPA and the FM were then compared and analyzed.

All data was maintained in Microsoft office Excel and statistical analysis carried out using Excel. Appropriate Statistical tools like tests of proportion and test of significance like Pearson's Chi Square test were applied.

3. Results

A total of 30 participants were included based on the inclusion criteria with 18/30 (60%) males and 12/30 (40%) females. But during the course of study we have lost 14 patients who have not reported for the follow up on either 3rd, 7th or 14th day. These participants were excluded from the final statistical analysis. Off the final tally of sixteen participants 10/16 (62.5%) were males against 6/16 (37.5%) females.

The participants and the results after FDA/EB staining with viable/non-viable mycobacteria ratio is as given in Table 1.

All the strains were also stained with conventional AFB staining which could detect the AFB in all the samples but viability studies using FDA/EB could predict the resistant nature. A total of 4/16 (25%) strains were found out to be resistant based on the ratio of Viable/Non-Viable Mycobacteria which remains static or raised from day 0 to 14. (Table 1)

All the participants' samples were sent for LiPA. When compared with the gold standard of Nucleic acid amplification Technology and Line Probe Assay (LiPA) for prediction of the sensitivity and resistance as shown in Table 2, the predictive utility of FDA/EB was found to be highly significant with Chi square value of 11.73 with 1 degree of freedom with p value of 0.000614 which is <0.001 (Table 2)

4. Discussion

Multidrug resistant mycobacteria causing pulmonary tuberculosis is on the rise causing treatment failure in the patients put on Cat I. The primary aim at RNTCP now a days is to send the sample on the very first day of diagnosis by sputum smear microscopy for GeneXpert and LiPA. This puts an additional burden on already overloaded reference laboratories catering to such samples. This study was carried out to look for alternative method of using FDA/EB staining and FM for predicting the resistance in the patients by detecting the viability of the mycobacteria in the subsequent samples provided by the patient.

In our study, out of the total 30 patients who gave their primary sample on day 0, only 16 patients followed up on 3rd, 7th and 14th day. Out of these 16, 10 were males (62.5%) and 6 were females (37.5%). The reason for small sample size in our study was short duration of study and

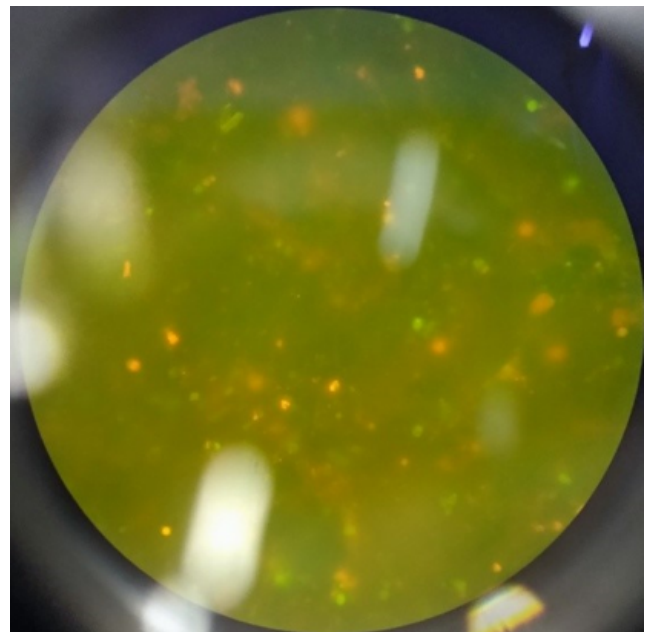


Fig. 1: Viable (Green) and Non-Viable (Redish Orange) Mycobacteria in prepared smear

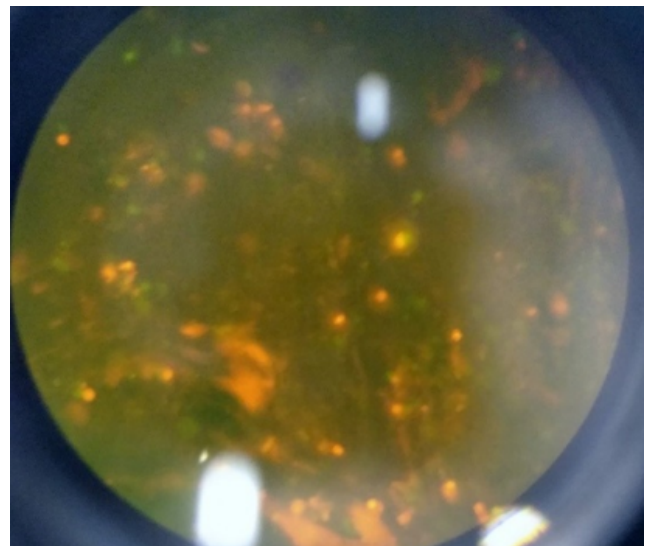


Fig. 2: Non-Viable Mycobacteria (Redish Orange) in prepared smear

patients coming from faraway places led to loss of follow up.

Out of the 16, 12 (75%) samples had ratio of viable and non-viable in decreasing order and hence were drug sensitive strain. 4/16 (25%) samples had static or increasing ratio of viable to non-viable mycobacteria and hence were resistant strains. In the study of 35 patients with newly diagnosed smear positive pulmonary tuberculosis in Peru by Datta et al, out of 35 patients, 31 had non MDR- tuberculosis and 4 had MDR- tuberculosis.⁵

Table 1: Results in all participants after FDA/EBstaining with viable/non-viable mycobacteria ratio and interpretation of the same in terms of sensitive and resistant strain

Participant Number	Day 0		Day 3		Day 7		Day 14		Interpretation
	Viable/Non-Viable	Ratio	Viable/Non-Viable	Ratio	Viable/Non-Viable	Ratio	Viable/Non-Viable	Ratio	
1	90/10		75/25	3	60/40	3	30/70	0.428	Sensitive Strain
2	85/15	5.66	68/32	2.12	58/42	1.38	12/17	0.70	Sensitive strain
3	75/25	3	80/20	4	78/22	3.54	79/21	3.76	Resistant Strain
4	95/5	19	85/15	5.66	88/12	7.33	80/20	4	Sensitive Strain
5	98/2	49	90/10	9	85/15	5.66	88/12	7.33	Resistant Strain
6	88/12	7.33	85/15	5.66	75/25	3	6/4	1.5	Sensitive Strain
7	92/8	11.5	82/18	4.55	40/20	2	38/12	3.66	Sensitive strain
8	85/15	5.66	80/20	4	88/12	7.33	92/8	11.5	Resistant Strain
9	75/25	3	70/30	2.33	68.32	2.12	13/12	1.08	Sensitive strain
10	90/10	9	75/25	3	60/40	3	30/70	0.428	Sensitive Strain
11	85/15	5.66	68/32	2.12	58/42	1.38	12/17	0.70	Sensitive strain
12	88/12	7.33	85/15	5.66	75/25	3	6/4	1.5	Sensitive Strain
13	98/2	49	90/10	9	85/15	5.66	88/12	7.33	Resistant Strain
14	95/5	19	85/15	5.66	88/12	7.33	80/20	4	Sensitive Strain
15	75/25	3	70/30	2.33	68.32	2.12	13/12	1.08	Sensitive strain
16	96/4	24	84/16	5.25	10/5	2	6/4	1.5	Sensitive strain

Table 2: Comparative evaluation of FDA/EB staining against Gold standard of LiPA for predictive utility of drug resistant TB.

	Sensitive by LiPA	Resistant by LiPA	Total	Chi Square value & p value	Interpretation
Sensitive by FDA/EB	11	1	12	$\chi^2=11.73$ P value=0.000614	Highly Significant
Resistant by FDA/EB	0	4	4		
Total	11	5	16		

The results were compared with the gold standard LiPA (line probe assay) and Nucleic acid amplification test, for which all the samples were sent. The predictive utility of FDA/EB was found to be significant (p value < 0.001) (Table 2). Similar results were obtained by Datta et al as viability changes were significantly greater for non-MDR tuberculosis than MDR tuberculosis after 3, 6, and 9 days of treatment (all $P < .001$) in their study.⁵

FDA/EB microscopy hence would detect the drug resistant cases and be used as tool for early detection and treatment. As also stated by Van Deun A et al that FDA staining increased the proportion of tuberculosis patients put on second-line treatment without receiving the standard first-line retreatment regimen.⁸

The higher cost of the Fluorescein diacetate and Ethidium Bromide dyes could be a drawback but still it would be cheaper than setting of LiPA and Nucleic acid amplification test. A study by Schramm B, Hewison C, Bonte L, et al stated that the patients are at risk of receiving an unnecessary prolonged or new treatment regimen in settings with limited or no access to M. tuberculosis culture. Culture is the only test that can identify viable bacilli, but it requires several weeks to report results and needs a high level of expertise and laboratory infrastructure.⁶ With this method, the burden of samples on health care workers of IRL would also reduce.

According to Lawn SD et al FDA viability testing might be widely employed in tuberculosis programs in resource limited settings as a means of early detection of drug resistant tuberculosis. Further relationship between viability microscopy and risk of tuberculosis transmission should be explored.² In a study by Datta S, Sherman JM, Tovar MA, Bravard MA, Valencia T, Montoya R, Quino W, D'Arcy N, Ramos ES, Gilman RH, et al. the infectiousness of TB was assessed by sputum microscopy with fluorescein diacetate.⁹

In terms of the study being undertaken as a short term studentship program, not much sample size could be achieved due to lack of time and there was a significant number of cases who lost to follow up. The operational research over a period of 2 years at the peripheral centers needs to be carried out with patient education and better follow up for more comprehensive results.

5. Conclusion

This study established the utility of fluorescent microscopy using FDA and EB to detect drug resistance in patients of pulmonary tuberculosis by evaluation of viable and non-viable bacilli in the sputum samples. The use of FDA-EB fluorescent microscopy will help reduce the cost as LiPA at private setting is expensive and will also lower the heavy sample load on the health care workers at IRL.

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7. Source of Funding

None.

8. Conflict of Interest

None.

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Author biography

Sushmita Mukherjee Student

Navinchandra M Kaore Professor and Head

Shilpa N Kaore Additional Professor

Surender Kaur Assistant Professor

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