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# Phage amplification technology for the rapid detection of *Mycobacterium tuberculosis* complex–clinical utility for the diagnosis of pulmonary tuberculosis

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**ABSTRACT**

*Rapid, low cost and sensitive methods play significant role in the treatment and management of Tuberculosis. Study includes utility of Phage Amplification Technology in parallel with in-house mpb-64 based PCR, microscopy, and BACTEC 460 Tuberculosis culture method for Mycobacterium Tuberculosis complex detection. Six hundred pulmonary specimens which include 300 sputum and 300 bronchial alveolar lavage (BAL) specimens), were considered for the study. The sensitivity, of AFB smear, PCR and FAST Plaque Tuberculosis (FPA) assay for the BAL were 50.9%, 90.2% and 88.67%respectively. While in case of sputum specimens the sensitivity, of AFB smear, PCR method and FASTPlaque TB assay test were 58.25%, 87.37%, 85.85% respectively. In conclusion, FPA proved to be sensitive, cheap, relative to the PCR and rapid than the culture. It gives result within 48 hours comparative to the culture which takes 2-6 weeks for the detection. In contrast to PCR, where it can not differentiate dead and live bacilli, FPA can detect live bacilli.*

**Keywords:** Phage amplification, Mycobacteriophage, Plaque, Mannose binding protein-64 gene.

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## INTRODUCTION

Tuberculosis (TB) is considered as one of the most serious public health problem and about one-third of the world's population is infected with Mycobacterium tuberculosis. There were an estimated 11.1 million (range, 9.6–13.3 million) prevalent cases of TB in 2008, equivalent to 164 cases per 100 000 population and an estimated 1.3 million (range, 1.1–1.7 million) deaths, including 0.5 million (WHO, 2009). Many publications have predicted approximately 1 billion new tuberculosis cases will be newly infected by the year 2020 (1, 2). The disease is especially prevalent in developing countries including India and Indian subcontinents accounting for the one-fifth of the global prevalence (3). Nearly 500, 000 thousand die from the disease, affecting the social and economical growth of the country (4).

In this view, effective and rapid detection methods for laboratory diagnosis and drug susceptibility testing of TB, that are suitable for implementation in low income countries, which bear the highest TB burden, are urgently needed for effective identification, treatment and control of the disease. Conventional diagnostic methods like AFB smear for detection of acid fast bacilli (AFB) has been frequently used for the diagnosis of tubercular bacterial infection in developing countries but it suffers with low specificity and variable sensitivity (5). Culture is currently regarded as the definitive method for the detection of viable mycobacterium which is more sensitive but time consuming and susceptible to contamination problems (6). Although new, more rapid and sensitive diagnostic methods have been

developed that are based on liquid culture techniques such as BACTEC TB system or molecular techniques such as PCR, they being costly have limited applicability in low resource settings like in developing nations (7).

The FASTPlaqueTB (FPA) assay also called phage amplification assay, developed by BIOTEC Laboratories Ltd and FIND (Foundation for Innovative New Diagnostics) and manufactured by BIOTEC Laboratories Ltd, is a novel application of phage amplification technology which allows the rapid detection of viable *M. tuberculosis* from respiratory specimens within 24 hours of specimen preparation (8). Mycobacteriophages, first isolated by Gardner and Weiser in 1947 are used as tools in diagnosis in this technique. The FASTPlaqueTB assay (also known as phage amplification assay) is a cost effective, rapid technique which is very popular in developing countries and is the only established tool which can detect viable Mycobacterium tuberculosis complex in sputum specimens within 48 hours. It is based on the infection of Mycobacterium tuberculosis with mycobacteriophage and its replication within host cells. These phages are mixed and allowed to infect the *Mycobacterium* cells present in clinical specimens (9). All the unadsorbed extra cellular phages are then inactivated using a virucidal solution (Virusol), thus only those survive which have infect the cells and continue their replication. Fast growing helper cells or sensor cells (*Mycobacterium smegmatis*) are mixed with the specimens on agar plate to detect the progeny bacteriophages an overnight incubation. These progeny phages infect, replicate and lyse these helper cells forming clear zones or plaques. These plaques on agar plate represent the number of viable tubercle bacilli in the original specimens. If there are no plaques, it indicates there are no bacteria (i.e. viable *M. tuberculosis cells*).

The performance of FPA has been studied worldwide that showed very good results; in our study we evaluated the performance of the FPA in clinical specimens and comparing its results with those from AFB smear, culture and conventional PCR (10-13).

## MATERIALS AND METHODS

### Clinical specimens

600 pulmonary (sputum, BAL, endo tracheal secretions, etc.) suspected specimens from different hospitals of Delhi/NCR region were investigated during the period of January to August 2009.Three early morning sputum specimens were collected in sterile plastic containers. The specimens were collected either at the hospitals or in the collection centres of Auroprobe Laboratory and kept at $4^{o}$C. The specimens were then carried out at the laboratory next day for further processing. This study was approved by Institutional Ethical Committee

### Specimens processing (Decontamination and Concentration of Specimens)

Decontamination was carried out to remove other bacterial flora, fungal and other contamination which was likely to be present in all the specimens, using N-acetyl-L-cysteine (NALC) / NaOH method with some minor changes (14). Decontamination solution was prepared by adding 0.5 % NALC (w/v; Sigma) to a 1:1 mixture of 4% NaOH and 2.9% sodium citrate. The specimens were centrifuged in a 15 ml or 50 ml sterile centrifuge tubes at 5000 rpm for 20 min. Supernatant was discarded gently and the sediment was resuspended. 200 µl of the sediments were taken for PCR and equal volume of decontamination solution was added into it and were incubated at room temperature for 20 min. The specimens were neutralized with sterile phosphate buffer (0.067 M, pH 6.8) and then centrifuged at 5000 rpm for 20 min. Supernatant was discarded to get the sediment up to 2 ml and mixed with 500 µl of sterile PB. 500 µl of the resuspended sediments were taken for culture and remaining solution was used for acid fast staining and FASTPlaqueTB assay.

### Microscopy

AFB smears were prepared dropping the resuspended processed specimen over glass slides; after drying smears were stained by ZN acid-fast stain (30 g basic fuchsin, 50 g phenol crystals, 200ml 95% ethanol and double distilled water to 1litre) and decolourized by HCl and ethanol at final concentrations of 3 and 95%.

### BACTEC 460 TB Culture

200 µl of decontaminated specimen was inoculated into a BACTEC 12B vial with 100 µl of PANTA, which is a mixture of 5 different antibiotics polymixin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin and incubated at $37^{0}$ C for 6 weeks. Reading was taken at the end of every week. AFB smears were prepared from the vials with growth index (GI>25) was recorded. For further differentiation between Mycobacterium tuberculosis and mycobacterium other than tuberculosis (MOTT), NAP (ρ-nitro-α-acetylamino-β-hydroxy-propiophenone) test was performed in BACTEC cultures (15).

**FASTPlaqueTB Assay (FPA)**

FPA was performed according to manufacturer's guidelines (BIOTECH Laboratories). Minimum of 20 plaques was used to interpret the results according to manufacturer's instructions (fig. 1).

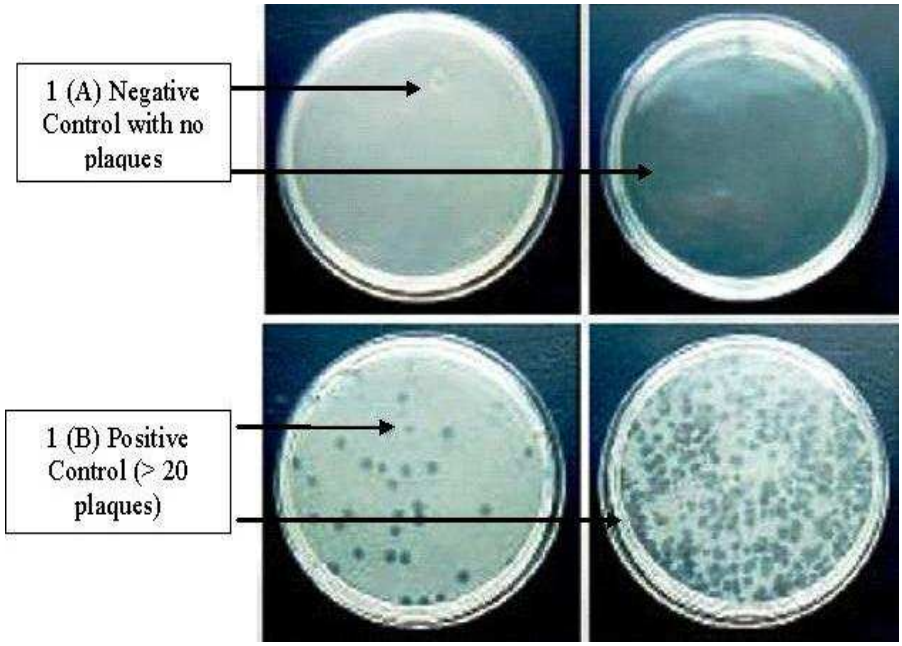


**Figure 1 (A) Negative Control with no plaques 1 (B) Positive Control (> 20 plaques).**

**PCR for Mycobacterium tuberculosis complex**

PCR was carried out for all the specimens. Silica adsorption based column method was used for the extraction of bacterial DNA and PCR was performed for the amplification of gene mannose binding protein 64 (mpb-64 gene) using primers Forward Primer (P1) 5'- TCCGCTGCCAGTCGTCTTCC-3' and Reverse Primer (P2) 5'-GTCCTCGCGAGTCTAGGCCA-3' (16, 17). Reaction mixture of 50 µl containing 10X PCR buffer (250 mM Tris HCl, 500 mM KCl), 0.2 mM dNTPS, 25µM primers, Taq DNA polymerase (3 units) and $Mg^{2+}$ ions (25mM) as $MgSO_4$ was prepared. 50µl of DNA template was added in the master mix and subjected for amplification on thermal cycler (Veriti, Applied Biosystems) with initial denaturation at 95°C for 5 min. 35 repetitive cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds and primer extension at 72 °C for 30 seconds with final extension at 72°C for 7 minutes. 240 base pair amplicon was observed when resolved on 1.5 % agarose gel.

## RESULTS

600 different pulmonary specimens were considered for the diagnosis of *Mycobacterium tuberculosis* by ZN staining method, BACTEC 460 TB, FPA, and conventional PCR and the clinical performance of FPA was compared with all the other mentioned techniques. 312 were sputum specimens and 302 were BAL specimens. Of the 614 specimens, 12 specimens got contaminated by other microorganisms and other two gave non interpretable results when tested by FPA. These were removed prior to analysis of the result. 205 of 600 (34.16 %) were BACTEC 460 TB culture positive out of which 102 were BAL specimens. 52(51%), 90 (91%) , 93 (89%) were AFB , PCR method and FPA positive. While for 103 sputum specimens which were positive by BACTEC460TB system, 60(59%) were positive for AFB smear, 90 (88%) were positive by PCR method & 96 (86%) by FPA. The sensitivity, specificity, positive predictive value(PPV), negative predictive value (NPV) for BAL specimens by AFB smear were found (50.9%, 100%, 100%, 79.83%) , while by PCR method it was (90.2%, 98.98%, 97.8%, 95%) and by FPA it was (88.7 %, 97.4%, 94.9%, 94.05%) respectively (Table 1). The sensitivity, specificity, positive predictive value(PPV), negative predictive value (NPV) for sputum specimens by AFB smear were 58.25%, 100%, 100%, 82.08%, while by PCR method it was 87.37%, 98.4%, 96.7%, 93.7% and by FPA it was 85.85%, 96.01%, 91.39%, 93.36% respectively (Table 2).

**Table 1. The sensitivity, specificity, positive predictive value and negative predictive values for different methods (BAL specimens, n-300)**

| Methods | True Positive | True Negative | False Positive | False Negative | Sensitivity | Specificity | Positive predictive Value (PPV) | Negative predictive Value (PPV) |
|---|---|---|---|---|---|---|---|---|
| AFB smear | 52 | 198 | - | 50 | 50.9 | 100 | 100 | 79.83 |
| PCR | 90 | 196 | 2 | 12 | 90.2 | 98.98 | 97.8 | 95 |
| Fastplaque Assay | 93 | 193 | 5 | 9 | 88.67 | 97.4 | 94.9 | 94.05 |

**Table 2. The sensitivity, specificity, positive predictive value and negative predictive values for different methods (Sputum specimens, n-300)**

| Method | True Positive | True Negative | False Positive | False Negative | Sensitivity | Specificity | Positive predictive Value (PPV) | Negative predictive Value (PPV) |
|---|---|---|---|---|---|---|---|---|
| AFB | 60 | 197 | - | 43 | 58.25 | 100 | 100 | 82.08 |
| PCR | 90 | 194 | 3 | 13 | 87.37 | 98.4 | 96.7 | 93.7 |
| FastPlaque Assay | 96 | 193 | 4 | 7 | 85.85 | 96.01 | 91.39 | 93.36 |

## DISCUSSION

As the number of TB patients are increasing day by day in India, thus the need for new rapid techniques play a major role in the rapid diagnosis and disease management (16, 17). Culture methods have been considered as gold standard for the detection of *M. tuberculosis*, BACTEC 460 TB culture system, Micro growth indicator tube (MGIT) 460TB system has been recommended as a valuable system with its high sensitivity and shortened duration of time required for the detection of mycobacteria 10 to 14 days. Nucleic acid amplification methods such as PCR provide an alternative approach in the detection of microorganisms and thus offer new possibilities for a more rapid and accurate diagnosis of tuberculosis . The numbers of false negative results were 25 with PCR method and 16 with FPA. We detected 5 false positive results with PCR and 9 with FPA. False positive results in PCR might have been due to an accidental contamination of the specimens during or before the PCR assay. False negative results obtained by PCR for culture-positive specimens may be explained by the presence of inhibitors of enzymatic amplification or by small number of *M. tuberculosis* strains unequally distributed in the test suspension.

Besides, false positive results may be due to insufficient addition or mixing procedure of virucidal solution resulting in failing to destroy the bacteriophages outside of the target cell and also it may be due to the contamination of the specimens by sensor cells. However, when false positive results detected, these may be due to incomplete destruction of exogenous phage by the virucidal solution possibly because of the protective effect of sputum components on the phage. FPA test requires viable bacilli so; there may also be false negative results due to long time interval between specimen collection and the beginning of the test or low number of live cells in the specimens. Besides, FPA test needs intact phage receptors on the viable cell surface for the phage attachment and replication. Phage inhibitory substances may be present in the specimens in different concentrations. If they are in high concentrations they may inhibit the phage-TB interaction. AFB smear sensitivity has been reported to vary from 30% to more than 70%. In our study, it was found as 50.9% for BAL specimens, 58.25% for sputum specimens and was within the range of the sensitivity reported. The sensitivity of the FPA test was found as 88.67% for BAL specimens and 85.85 % for sputum specimens. AFB smear can detect $10^3$-$10^4$ bacilli/ml bacteria in a specimen whereas FPA is able to detect 100-300 bacilli/ml. So, the sensitivity of FPA is higher than AFB smear method. The sensitivity and specificity of FPA were in agreement with PCR. These data suggest that FPA can be used as a diagnostic test for pulmonary tuberculosis. The test may be used in conjunction with sputum smear microscopy to detect additional cases that would be missed by smear alone. FPA relies on basic microbiologic techniques. Specialized equipment is not needed to perform the test and to evaluate the results. It is easy to perform in any laboratory, and actually helps the laboratories that use conventional manual culturing methods. Rapid diagnosis, high sensitivity and no special instrument requirement are the advantages of FPA. However risk of contamination during the test and the problems with the counting the plaques are disadvantages of this test.

## CONCLUSION

For the better treatment of the tuberculosis the most important point is early diagnosis, rapid, novel and cost effectiveness of an assay, especially in developing nations. FPA cost 400- 500 per test as compared to PCR and culture which is three to four times costlier. In low resource settings, phage-based assays can be directly used on

pulmonary specimens as they show high accuracy, they have the potential to improve the diagnosis and management. We conclude that the FPA demonstrates satisfactory clinical performance in terms of sensitivity and specificity.

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