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Evaluation of a new Phage Amplification technology for rapid diagnosis of Tuberculosi

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~ Abstract

PURPOSE: Rapid diagnosis of tuberculosis is essential to initiate timely and appropriate treatment to curb the spread of this potentially life threatening disease. The purpose of this study was to evaluate a phage amplification technology viz., FASTPlaque TB,™ for the diagnosis of tuberculosis. METHODS: We evaluated the clinical utility of this new assay by analyzing 50 respiratory and 40 non-respiratory specimens, using FASTPlaque TB™ kit (Biotec Laboratories, UK) and the performance was compared with TB Bactec 460 semi-automated liquid culture system and conventional LJ culture method. RESULTS: In case of respiratory specimens phage assay gave good specificity (100%) compared with TB Bactec whereas with respect to LJ method the sensitivity and specificity were 93.1% and 88.2% respectively. In case of non-respiratory specimens comparison of results obtained by phage assay showed sensitivity of 90.9% and specificity of 88.8% with respect to TB Bactec and 87.5% and 93.8% with respect to LJ method. CONCLUSIONS: We believe that this new low cost assay may have widespread applicability, especially in developing countries, due to its manual format and rapid reporting of results.

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Tuberculosis (TB) continues to be one of the leading infectious causes of death in the world today. It affects one-third of the world's population, of which 95% is in the developing countries where resources are limited.[1] In India, 13 million are infected and diseased, 3.5 million are positive for acid fast bacilli (AFB) with 2.2 million new TB cases being added every year.[2] The definitive diagnosis of TB continues to depend on microscopy and culture. Smear microscopy remains the mainstay of TB diagnosis in developing countries, but suffers from low specificity, and variable sensitivity.[3] Laboratory cultivation of *Mycobacterium tuberculosis* is much more sensitive, but it is time consuming and susceptible to contamination problems.[4] Rise in TB statistics and recent outbreaks of multidrug resistant (MDR) TB have heightened the importance of rapid diagnosis of this disease. Molecular methods for detection of TB are proving rapid and sensitive, but the high cost of these methods and requirement for sophisticated equipment currently renders them inappropriate for routine use in many countries with a high burden of disease.[5] Hence, any test broadly acceptable to the global TB diagnostic community needs to be cost effective, accurate, simple and easy to implement within the current infrastructure. This challenge has prompted scientists to reconsider the use of mycobacteriophages as tools in diagnosis and drug susceptibility testing. Gardner and Weiser isolated the first mycobacteriophage in 1947 and since that time over 250 phages have been identified.[6],[7] The recent upsurge of drug-resistant bacterial infection has prompted fresh interest in the field of phage therapy but, unfortunately, attempts to use lytic phages therapeutically during tuberculosis infection have so far failed to elicit cure in experimentally infected animals.[7] Instead, the use of these mycobacteriophages in investigative studies of mycobacteria has become widespread and recently, their potential as tools for drug susceptibility testing was reported.[1],[8],[9] FASTPlaque TB,TM a new rapid test for diagnosis of TB, was launched in year 2000 by Biotec Laboratories Ltd. FASTPlaque TB™ is a novel, patent protected, phage amplification technology that has been developed for rapid detection and enumeration of *M.tuberculosis* complex from respiratory specimens. This method uses specific mycobacteriophages (viruses that infect *M.tuberculosis* complex) to detect the presence of viable TB bacilli in the clinical specimen.[3],[10] Mycobacteria are mixed with phages, which are allowed to adsorb and infect the cells. All unadsorbed extracellular phages are then inactivated using a phagicidal chemical (virucide); while the phages which have infected the viable TB bacilli remain protected and continue to replicate. After replication the progeny bacteriophages are released and detected by mixing with fast growing non-pathogenic helper cells (*M.smegmatis* sp) on an agar plate. The mycobacteriophages in turn infect, replicate and lyse these helper cells and lysis is detected as plaques (clear zones). The number of plaques visualized from a given sample is related to the number of viable tubercle bacilli in the original sample.[8],[11],[12] In *M.smegmatis* the lytic cycle is completed within 90 minutes whereas lysis takes approximately 13 hours in *M.tuberculosis* complex thereby making results available rapidly in terms of plaques.[7] In this study, we have evaluated the clinical utility of phage assay, using FASTPlaque TB kit, by comparing the results with clinical data, smear microscopy, and culture methods.

~ Materials and Methods

Patients

The study comprised of 50 respiratory (sputum, bronchoalveolar lavage and endotracheal secretion) and 40 non-respiratory (pleural fluid, CSF, cold abscess, lymph node, pus, urine etc.) specimens collected at PD Hinduja National Hospital and Medical Research Center. The clinical history, symptoms, radiological, histopathological, other laboratory findings, and past and current details of medication (if taken) of all these subjects were collected.

Decontamination and concentration of specimens

All specimens, which were likely to contain normal or transient bacterial flora, were decontaminated by standard N-acetyl-L-cysteine-NaOH method,[13] while specimens collected from sterile sites were centrifuged and sediment was used to perform the FASTPlaque TB test. Decontaminated and concentrated sediment was resuspended in 2 mL of sterile 0.67 M phosphate buffer (pH 6.8) and used for phage assay and cultures by TB Bactec and Lowenstein Jensen (LJ) methods.

Microscopy

Smears were made for all the clinical specimens studied and stained with Ziehl-Neelsen carbol fuchsin (ZN) staining method[13] for AFB.

FASTPlaque TB Assay

The principle of the FASTPlaque TB assay is shown in [Figure - 1].

The assay was carried out by using FASTPlaque TB™ kit (Biotec laboratories, UK). All respiratory specimens were processed according to the instructions given by the manufacturer whereas minor modifications were made in the procedure while processing non-respiratory specimens as this kit was not recommended for non-respiratory specimens. Both Positive and negative controls were also included in the assay and tested as per the manufacturer's instructions. Negative control contained 1 mL of plain FASTPlaque TB broth, whereas three positive controls were prepared by serial dilution (10-2,10-4,10-6) of *M.smegmatis* respectively. These controls were included to assess the integrity of the phage and effectiveness of the phagicidal agent. For the assay 1 mL of decontaminated and concentrated sediment was mixed with 1mL of FASTPlaque TB broth and incubated at 370C overnight to enrich viable TB bacilli present in the sample. In case of non-respiratory specimens enrichment period was increased to 48 hours. After enrichment 100 mL of mycobacteriophage solution was added and incubated for further 1 hour to allow infection to take place. Then 100 mL of virucide solution was added for destruction of all bacteriophages, which have not infected host cells and incubated at room temperature for 15 min. Then 5 mL fast plaque TB medium was added to neutralize excess of virucide, followed by 1 mL of helper cells. After mixing thoroughly it was added to the petridish and overlayered with 5 mL of molten agar. On pouring, plates were rotated several times, both clockwise and counterclockwise. Plates were allowed to set and they were incubated at 370C and number of plaques was counted after overnight incubation. A cutoff of 20 plaques was used to interpret the results as recommended by the manufacturer [Figure:2].

TB Bactec 460

0.5 mL of processed specimen was inoculated into Bactec 12 B vial supplemented with PANTA (a mixture of 5 different antibiotics polymyxin B, amphotericin B, nalidixic

acid, trimethoprim and azlocillin) and incubated at 370C. Reading was taken daily for first 3 weeks and thereafter once a week for culture positivity till the end of 6 weeks. AFB smear was made from vials with GI>30 and further identification of mycobacteria grown in Bactec cultures was done by NAP (para-nitro-alpha-acetylamino-beta-hydroxy-propiophenone) test.[14],[15]

LJ method

0.5 mL of processed specimen were inoculated on 2 slants of Lowenstein-Jensen (LJ) egg medium and incubated at 370C. The slants were inspected every day for first week and then weekly for 10 weeks. All culture positives were confirmed by ZN microscopy and further identification was done by standard biochemical tests.[13]

Statistical analysis

The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for phage assay were calculated by comparing with the AFB smear, TB Bactec, LJ method and also by comparing with the clinical evidence of disease. The following formulae were used for calculations. Sensitivity was true positives/(true positives + false negatives) x 100; specificity was true negatives/(true negatives + false positives) x 100; PPV was true positives/(true positives + false positives) x 100; and NPV was true negatives/(true negatives + false negatives) x 100.

~ Results

The comparison of phage assay with AFB smear of both respiratory and nonrespiratory specimens is shown in [Table - 1]. Of the 50 respiratory specimens results concurred in 41 cases whereas discrepancy was noticed in nine cases. Of these nine AFB smear positive and phage assay negative samples, six were identified, as Mycobacteria Other Than Tuberculosis (MOTT) whereas remaining three were late culture positives. A sensitivity of 90.6% and excellent specificity of 100% was observed in case of respiratory specimens.

In case of non-respiratory specimens results obtained by both methods were comparable in 25 cases whereas disparity was observed in 15 cases, of which only one was identified as MOTT. In the 13 AFB smear positive and phage assay negative cases, all patients were found to be on treatment. One AFB smear negative phage assay positive case was clinically diagnosed as a case of TB meningitis. In case of respiratory samples, sensitivity, specificity, PPV and NPV were 61.8%, 81%, 68.1% and 23.5% respectively. [Table - 2] shows comparison of phage assay with culture techniques (TB Bactec and LJ). Of the 50 respiratory specimens, 39 comparable results were obtained by Bactec and phage assay showing sensitivity of 90.6% and specificity of 100%. Of the 11 discrepant results eight were identified as MOTTs and three were late culture positives. Further comparison of phage assay with culture on LJ showed 42 comparable results. Of six LJ positive and phage assay negative specimens four were MOTTs and two were late culture positives. Two phage assay positive and LJ negative specimens were identified as *M.tuberculosis* complex by TB Bactec. The overall sensitivity and specificity of phage assay with respect to LJ was 93.1% and 88.2%.

In case of non-respiratory specimens comparison of Bactec with phage assay indicates discrepancy in four cases. Two Bactec positive phage assay negative specimens were

late culture positives. Among two phage assay positive (CSF and urine) Bactec negative one was clinically diagnosed case of TB meningitis and in the other case there was a mixed infection with *M.tuberculosis* complex and *Mycobacterium* spp. showing two different types of colonies on LJ. However, only *Mycobacterium* spp. was grown in Bactec where as *M.tuberculosis* complex was picked up by phage assay. Further comparison of phage assay with LJ shows discrepancy in four cases of which three late culture positives were negative by phage assay. One phage assay positive specimen was negative by LJ. The overall sensitivity, specificity were 90.9% and 88% with respect to Bactec and 87.5% and 88.2% respectively with respect to LJ.
We further compared phage assay results with clinical evidence of disease (TB) as indicated in [Table - 3]. All 90 patients were divided into two groups viz., disease present and disease absent. The presence of disease was determined by positive AFB smear, positive culture results, or those with history of TB, clinical, radiological and other laboratory findings suggestive of TB. Of the 50 respiratory samples studied 45 cases were correctly diagnosed by phage assay whereas discrepancy was observed in five cases. All these five were on treatment of which three were late culture positives. The overall sensitivity and specificity were 85.3% and 100% respectively. In case of non-respiratory samples 15 clinically diagnosed cases of tuberculosis were negative by phage assay decreasing the sensitivity to 59.5% and NPV to 16.6%. Of these 15, 14 patients were on treatment, and three of these were late culture positive.

~ Discussion

Rapid and accurate diagnosis allows proper management of a disease. Current methods of diagnosis of tuberculosis are either time consuming or costly, therefore, a rapid, reliable, simple and cost effective method would be highly desirable, especially in developing countries where prevalence of tuberculosis is high. The phage assay is a simple technique, which does not require any expensive instrumentation and can be used in most of the routine mycobacteriology laboratories. An additional advantage is the safety during the assay procedure as large percentage of the bacilli are rendered noninfective by mycobacteriophages. This is in contrast to culture techniques where a substantial increase in the number of infective particles is observed.[9]
Phage assay has a short detection time of 24 - 48 hours compared to LJ and TB Bactec. Results are available in terms of plaques and are easy to interpret. In our study, plaques varied in number from 35-300. In majority of highly positive cases (3+ and 4+) by smear more than 300 plaques were observed. Variations in plaque number could be attributed to, the number of viable TB bacilli present in the clinical specimen, and presence of small clumps which appear to protect the bacilli from phage infection thereby affecting the kinetics of phage infection.
In this study, the clinical utility of phage assay has been evaluated using FASTPlaque TB kit. Of the 50 respiratory specimens, 38 were positive for AFB by smear, 32 were isolated as *M.tuberculosis* complex by TB Bactec, 29 by LJ medium, phage assay respectively showing concordance with culture methods. Of the 40 non-respiratory specimens, 35 were positive by smear of which 22 were isolated as *M.tuberculosis* complex by TB Bactec and by phage assay whereas 24 were isolated as *M.tuberculosis* complex by LJ medium. In case of non-respiratory specimens, though a good sensitivity and specificity was observed with respect to culture methods, discrepancy was seen

when compared with AFB smear.

As indicated in [Table - 1], there were 23 culture positive and phage assay negative specimens (9 respiratory and 14 non-respiratory). Of these, six (3 respiratory and 3 non-respiratory) patients were on anti-TB treatment and late culture positive. In Bactec the growth was seen almost after 4-6 weeks and on LJ medium after 6-8 weeks, demonstrating low number of viable organisms in the sample. In case of non-respiratory specimens of the remaining 11 discrepant smear positive, one was MOTT and 10 were negative by culture (LJ and Bactec). All these 10 patients were also on anti TB treatment. Possible explanation for all these “smear positive phage assay negative” cases could be the low cell numbers (well below the analytical sensitivity of the assay) due to effective anti-TB therapy. This suggests that the assay could be used as an important tool to monitor the treatment success as it detects only viable bacilli. Phage assay showed decreased sensitivity as compared to AFB smear [Table - 1] which also picked up non-viable TB bacilli.

In case of respiratory specimens, of the eight MOTTs isolated by TB Bactec [Table - 2], four grew on LJ medium, however, in case of non-respiratory specimens only two MOTTs were isolated by both Bactec and LJ. Of these 10 MOTTs only one was found positive by the phage assay. Further investigation of this case on LJ revealed the presence of two different types of colonies indicating the presence of a mixed infection of *M.tuberculosis* complex with MOTT. This highlights the potential of phage assay to pick up only *M.tuberculosis* complex even in the presence of other contaminants (*Mycobacterium* spp.) thus underlining its specificity for *M.tuberculosis* complex.

On further comparing the phage assay results with clinical evidence of disease [Table - 3] a specificity of 100% was observed for both respiratory as well as non-respiratory specimens showing a very low incidence of false positive results.[16] But as the number of non -TB cases studied here are less (16 respiratory and only 3 non-respiratory) more number of non-TB cases should be tested to confirm this finding. Though the sensitivity in case of respiratory samples was 85.3%, a very low sensitivity of 59.5% was observed for non-respiratory specimens. The possible explanation for this is that 40% (16/40) of the non-respiratory specimens included in this study were negative by both culture methods indicating less than 10 viable mycobacteria/mL (as culture can detect 10-100 organisms/mL). As the analytical sensitivity of the phage assay is 100-300 bacilli/mL, all these specimens were negative by phage assay.[16] The assay therefore is not useful in direct detection of *M.tuberculosis* using paucibacillary specimens containing less number of organisms and the clinical information should be taken into consideration while interpreting the results. In our study, better results were observed in case of non-respiratory specimens by increasing the enrichment period from 24 to 48 hours. Perhaps increasing initial number of organisms present in the sample by culturing it for few days in liquid media may help in increasing the sensitivity of non-respiratory specimens.

The sensitivity, specificity of FASTPlaque TB test with respect to culture and clinical evidence of disease in case of respiratory specimens were excellent and also in agreement with the results obtained from studies performed by Mole *et al*.[3] Due to the non availability of data on non-respiratory specimens, comparison of our results with other studies could not be done.

We conclude that the phage assay is simple to perform and inexpensive as it does not require any sophisticated or dedicated equipment. Results are available within 48-72

hours allowing earlier reporting and aiding appropriate therapeutic decision making. It is highly specific for MTB complex and can be used as a rapid screen for TB in case of respiratory specimens. As it detects only viable TB bacilli it might be used as a sensitive tool for monitoring the treatment success. Further additional research is required in order to apply it for direct detection of *M.tuberculosis* complex from paucibacillary specimens.

Figures and Tables

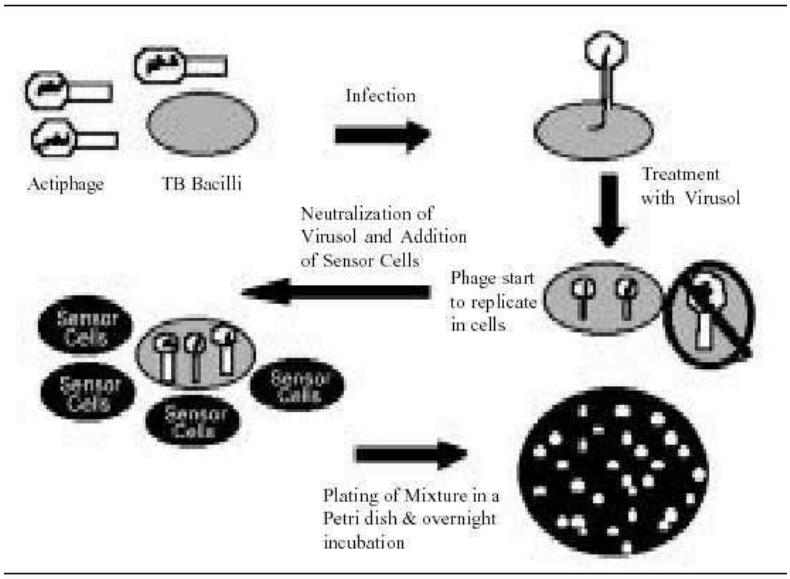

Figure 1 : Principle of FASTPlaque TB assay

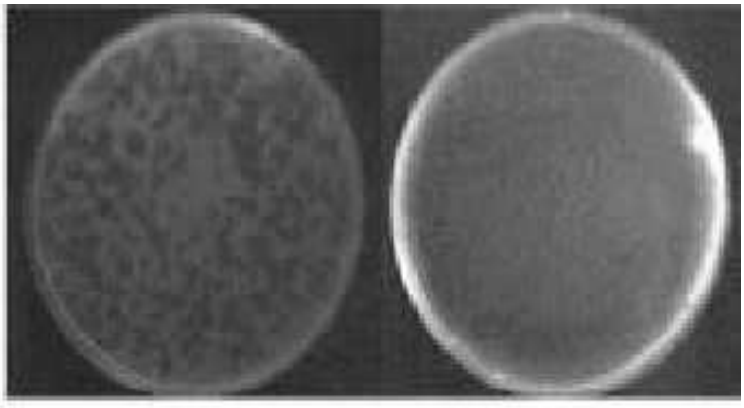

Figure 2 : Positive (>20 plaques) Negative (No plaques)

Table 1 : Comparison of Phage assay with AFB smears

	Respiratory Samples (n = 50)		Non-respiratory Samples (n = 40)	
AFB Smear	Phage positive	Phage negative	Phage positive	Phage negative
Positive	29	06*+03	21	1*+13
Negative	00	12	01	04

Table 2 : Comparison of Phage assay with TB Bactec and LJ method

	Respiratory Samples (n = 50)		Non-respiratory Samples (n = 40)	
	Phage positive	Phage negative	Phage positive	Phage negative
Bactec positive	29	08*+03	20	02
Bactec negative	00	10	02	16
LJ positive	27	04*+02	21	03
LJ negative	02	15	01	15

Respiratory samples

With respect to Bactec: sensitivity-90.6%, specificity-100% PPV-100%, NPV-76.9%

With respect to LJ: sensitivity-93.1%, specificity-88.2%, PPV-93.1%, NPV-88.2%

Non-respiratory samples

With respect to Bactec: sensitivity-90.9%, specificity-88.8% PPV 90.9%, NPV-88.8%

With respect to LJ: sensitivity-87.5%, specificity-93.8%, PPV-95.5%, NPV-83.3%

*MOTT

Table 3 : Comparison of Phage assay with clinical evidence of disease

	Respiratory Samples (n = 50)		Non-respiratory Samples (n = 40)	
	Phage positive	Phage negative	Phage positive	Phage negative
Disease present	29	05	22	15
Disease absent	00	16	00	03

Respiratory samples: Sensitivity-85.3%, Specificity-100%, PPV-100%, NPV-76.2%
Non-respiratory samples: Sensitivity-59.5%, Specificity-100%, PPV-100%, NPV-16.7%

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