Original Article

A STUDY OF PHAGE BASED DIAGNOSTIC TECHNIQUE FOR TUBERCULOSIS

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Summary

Background: Tuberculosis (TB) is a leading infectious disease in India.Diagnosis of TB has always been a problem due to slow rate of growth of *Mycobacterium tuberculosis*. In this study we have compared the conventional tools for diagnosis of TB with the new Fast Plaque TB ™.

Material and Methods: Two hundred and twelve clinically suspected cases of TB were enrolled for the study. Three consecutive early morning sputum samples were collected from each patient. Sputum smears were examined after staining with ZN stain and the sputum samples were later subjected to culture and phage assay.

Results: It was seen from the study that out of the total 212 cases, 106 were phage positive and 106 were phage negative. Sensitivity of the phage test with respect to AFB smear is 94.34% and specificity of the phage test is 93.88%. A total of 120 specimens grew on LJ media, of which 112 were *Mycobacterium tuberculosis*, 2 were *Mycobacterium Kansasii*, 4 were *Mycobacterium avium complex* and 2 grew *Mycobacterium fortuitum* group. Out of these 120 specimens which grew on LJ, 104 were also positive for phage assay. All the 8 Non-Tubercular Mycobacteria were negative by the Fast Plaque Assay. Out of the 92 which were negative on LJ, 2 were positive by phage assay. Sensitivity and specificity of phage assay with respect to growth on LJ was 92.86% and 97.83% respectively.

Conclusion: The study showed that phage assay is a rapid, reliable and cost effective method in diagnosing pulmonary tuberculosis from clinical samples. **[*Indian J Tuberc 2007; 54:36-40*]**

Key words: Tuberculosis, *Mycobacterium tuberculosis*, Bacteriophage assay

INTRODUCTION

Tuberculosis (TB) is a leading health problem worldwide and remains one of the leading causes of death from infectious diseases. An estimated 2 billion people (i.e., one third of the world's population) are infected with *M. tuberculosis*. Each year, approximately 9 million people suffer from the disease, and approximately 2 million die as a result.[1] Tuberculosis kills more adults in India than any other infectious disease. More than 1,000 people a day or one in every minute die of TB in our country.[2]

The prevalence of all forms of TB in India is estimated to be 5.05 per thousand, prevalence of smear positive cases 2.27 per thousand and average incidence of smear positive cases is 84 per 100,000 annually.[3] The incidence of TB is expected to increase substantially worldwide because of the interaction between TB and human immunodeficiency virus (HIV) /AIDS epidemic.

Nearly 1.8 million Indians get infection every year. Everyday, about 5000 people develop the disease and around 1000 die.[4] In India TB kills more in the younger age group thus compounding to the economic loss of the country. The direct cost of the disease in India annually is estimated at US$300 million; the annual indirect cost is US$3 billion.[4]

In our country, with a high prevalence of Tuberculosis, diagnosis is mainly based on the conventional methods like clinical assessment, radiology, sputum microscopy and culture in Lowenstein Jensen (LJ) media. New diagnostics approaches, including nucleic acid amplification, antibody detection, liquid culture, cellular immune response, antigen capture, and chemical and physical

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detection tests have been developed.[5] Many molecular methods have been developed for direct detection, species identification, and drug susceptibility testing of mycobacteria.[6] These require expertise and finance, and are not easily affordable in low income countries. The sensitivity of smear microscopy has been between 20%-80% in culture confirmed TB cases.[7] Though smear microscopy can detect positive cases if properly performed, it can miss quite a number of paucibacillary cases. The quality of results with smear microscopy is heavily dependant on the workload, skill and motivation of the technician reading the slides.[8] Culture techniques are available but the time required and negative results in paucibacillary cases are important limitations.[9] Chest X-ray is commonly used to aid the diagnosis of TB. However, since radiological changes are not specific for TB and do not always reflect active disease, over-reliance on chest X-ray can lead to misdiagnosis.[8] Therefore, there is need for a rapid, reliable and sensitive method for diagnosis of pulmonary tuberculosis so that early treatment can be started and the disease can be contained.

Fast Plaque TB ™ is a rapid manual bacteriophage based test to detect viable *Mycobacterium tuberculosis* (*M.tb*) in clinical specimens. The technique uses a mycobacteriophage which is able to infect and replicate in slow growing pathogenic strains e.g *M.tb*, *M. ulcerance* and also in some rapidly growing strains as *M. smegmatis*. Mycobacteriophages have the potential to become useful tools in the diagnosis of TB, as they are specific for mycobacteria and only replicate in, and hence detect, viable cells. Phage-based techniques involve simple manual manipulations and yield results rapidly.

In this technique, phages are added to the decontaminated sputum samples so that viable target bacilli are infected. A potent virucide is added which rapidly destroys any bacteriophage outside the target cells, without affecting phages inside the bacilli. These surviving bacteriophages replicate inside the tuberculer bacilli and lyse the bacteria in order to release the progeny bacteriophage. Virucide added earlier is neutralized and non-pathogenic rapidly growing mycobacterium which is also susceptible to the bacteriophage is then added. This is then plated on agar mixture as a lawn.The rapidly growing mycobacterium grows overnight and if it is infected with the phage, plaques are formed which indicate that viable tuberculer bacilli were present in the original specimen.

In this study, we have tried to compare the new Fast Plaque TB ™ with the conventional methods for diagnosing pulmonary tuberculosis i.e. direct microscopy as per RNTCP and culture on Lowenstein-Jensen (LJ) media.

MATERIAL AND METHODS

Two hundred and twelve suspected cases of tuberculosis attending DOTS Centre at UCMS & GTB Hospital, Delhi, were enrolled for the study. Under DOTS, tuberculosis "Suspect" patients are those who present with symptoms and signs suggestive of TB, in particular cough of long duration.

Three consecutive early morning sputum samples from all 212 patients were examined. These were collected in a clean leak proof labelled sterile container. First, smear was prepared and stained by Ziehl-Nielsen (ZN) stain. These stained smears were examined for the presence of acid fast bacilli (AFB) and graded as per RNTCP recommendations. These samples were then decontaminated and concentrated as per manufacturer's specifications. The assay was carried out by using Fast Plaque TB™ kit. For the assay, decontaminated and concentrated sediment was mixed with FPTB Medium Plus and incubated at 37^0C overnight to enrich viable TB bacilli present in the sample. After enrichment, Actiphage™ solution was added and incubated for further one hour to allow infection to take place. Then Virusol™ solution was added for destruction of all bacteriophages, which have not infected host cells and then incubated at room temperature for 15 minutes. FPTB Medium Plus was again added to neutralize excess of virucide, followed by Sensor™ cells. 5 mL of FPTB molten agar was poured to pre-labelled petridish and to it was added the above reaction mixture. The plates were mixed well and allowed to set at room temperature. Then they were incubated at 37^0C

overnight. Next day results were recorded as plaque formation. Plaque formation indicated presence of viable bacilli in the original sample. Results were interpreted as positive if ≥ 20 plaques were present and 0-19 plaques signified negative results.

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The deposits formed after concentration of the sputum samples were also inoculated on LJ media slope in duplicate. These were examined weekly for growth. Any growth was checked by ZN staining. Identification was done on the basis of rate of growth, colony morphology, pigment production, biochemical tests like niacin production, aryl sulphatase, catalase production, nitrate reduction, growth on PNB, tween hydrolysis and TCH susceptibility test as per CDC manual ,1985.

RESULTS

Out of the total 212 tuberculosis "Suspect" patients, 110 were male and 102 were female (male: female ratio – 1:0.92). The age of the patients ranged from 10-60 years with maximum patients in age group 20-39 years (84, 39.62%) followed by 40-59 years (78, 36.80%).

Table 1 represents distribution of tuberculosis patients as per DOTS programme. Accordingly there were 124 "Cases" of tuberculosis. 112 of these were "Definite" cases and rest 12 were not as 2 were diagnosed by radiology and 10 were only one smear and radiologically positive.

Definite TB cases under DOTS are those with positive culture for *Mycobacterium tuberculosis* and in countries where culture is not routinely available, two sputum smears positive for AFB is also considered a "Definite" case.

In all, 104 of these "Definite" TB cases were both smear and culture positive and 8 were only culture positive but smear negative.

Out of 212 Tuberculosis "Suspect", 114 were found to be positive by sputum smear examination.10 out of these were one smear positive and supported by radiology. 98 were sputum smear negative. Out of these, 8 grew on culture and 2 were supported by radiology, raised ESR, etc.

Table 2 shows results of Fast Plaque Assay with respect to AFB smear positivity. Of 212, 106 were phage positive and rest 106 were phage negative. Out of 114 smear positive cases, 100 were phage positive and 14 were phage negative; 8 of which were Non Tubercular Mycobacteria (NTM).

Table 1: Case distribution as per DOTS case definition.

No. of cases of tuberculosis (124)		Definite cases (112)		Smear positive Pulmonary Cases (114)		Smear negative Pulmonary Cases (10)	
New cases	Relapse cases	Culture +ve & 2 smear +ve	Culture +ve & smear -ve	Culture+ve & 2 Smear+ve	1 smear +ve & X-ray +ve	Culture+ve & Smear-ve	Only X-ray+ve
108	16	104	8	104	10	8	2

Table 2: Results of Fast Plaque Assay with respect to AFB smear positivity

	Phage positive	Phage negative	Sensitivity (%)	Specificity (%)
AFB smear positive (114)	100	6+8(NTM)	94.34	93.88
AFB smear negative (98)	6	92		
Total (212)	106	106		

Table 3: Comparison of growth on LJ with phage assay.

	Phage positive	Phage negative	Sensitivity (%)	Specificity (%)
LJ positive (120)	104	8+8(NTM)	92.86	97.83
LJ negative (92)	2	90		

Amongst the 98 smear negative samples, 6 samples were positive by phage assay.

The sensitivity of the phage test with respect to AFB smear positivity was 94.34% and specificity was 93.88%. The positive predictive value was 94.33% and negative predictive value was 93.88%.

All 212 samples were cultured on LJ media and 120 grew acid fast bacilli (56.60%) which were confirmed by ZN smear and biochemical tests. These included 112 *M.tuberculosis* and 8 NTM isolates. These were 2 *M.Kansasii*, 4 *M.avium complex* and 2 *M.fortuitum* group. There were 10 sputum negative pulmonary TB cases, 8 of which grew *M.tuberculosis* on LJ media. They also had raised ESR.

Table 3 compares result of culture positivity with phage assay. It was seen that of a total 120 culture positive samples, 104 were also phage positive. Rest 16 were phage negative of which 8 were NTM. 92 samples did not grow on LJ media and out of these 2 were positive by Phage assay. Thus the sensitivity and specificity of phage assay with respect to growth on LJ media was 92.86% and 97.83% respectively. The positive predictive value was 98.11% and negative predictive value was calculated at 91.84%.

DISCUSSION

Diagnosis of TB has been a problem due to slow growth of the organism. This hampers in treatment of cases, thus increasing the mortality and morbidity of the disease. Smear microscopy is simple and most rapid procedure currently available to detect AFB in clinical specimens. The limit of detection with this method is that it requires at least $5x10^3$ bacilli per ml of sputum.[10] The major drawback of growing mycobacteria in conventional media is its slow growth which requires an incubation period of at least 4 weeks. Thus there is need for a rapid, reliable and sensitive method for the diagnosis and treatment of the disease. Phage assay is a simple technique which does not require any expensive instrumentation and can be used in most routine mycobacteriology laboratory.

Phage assay has short detection time of 24-48 hours compared to conventional growth on Lowenstein Jensen media. Results are easily available in terms of plaques and easy to interpret. In our study, plaques varied in number from 25 to more than 300. In majority of highly positive cases by smear, more than 300 plaques were seen as confluent lysis on agar plate. Variation in plaque number can be attributed to number of viable bacilli present in sputum samples.

In this study, samples were taken from DOTS centre in our hospital. In DOTS, AFB smear is taken as diagnostic test and AFB positive patient is started on anti-tubercular treatment (ATT) with assumption that patient has *M.tuberculosis* infection. On comparison of AFB smear with Fast Plaque Test, (table 2) it was found that out of 212 samples, 100 were both AFB smear and phage positive. 14 were AFB smear positive and phage test negative; 8 of them later on grew NTM. Phage test is highly specific for *M. tuberculosis* complex and so could not detect NTM. Two samples not detected on phage test were re-treatment case, already on ATT. Either ATT drugs interfered with phage test or else bacilli were non-viable and phage test detects only live bacilli.

There were 6 cases which were phage test

positive and smear negative. These 6 phage positive cases also later grew *M.tuberculosis* on culture. Phage assay has analytical sensitivity of 100 bacilli, so it detected paucibacillary specimens, which were missed on AFB smear alone.

The sensitivity of phage test with respect to AFB smear positivity was 94.34% and specificity was 93.88% in our study. S. Shenai et al[11] recorded a similar result with a sensitivity of 90.6% and specificity of 100%. Phulputo et al[12] reported sensitivity and specificity at a low of 54.16% and 83.33% when Fast Plaque was compared with smear positivity. Muzarraf et al[13] recorded sensitivity and specificity of 87.4% and 88.2% respectively while Albert et al[14] recorded sensitivity and specificity of 86.8% and 83.8% respectively.

In our study the sensitivity and specificity of phage assay when compared to growth on LJ media (Table 3) were 92.86% and 97.83% respectively. Muzarraf et al[13] showed sensitivity and specificity of 81.6% and 97.7% respectively, which is comparable to our study. Phulputo et al.[12] recorded sensitivity and specificity of 86.23% and 96.42% respectively while Shennai et al[11] recorded 93.1% and 88.2% in their study. A sensitivity and specificity of 58.3% and 99.1% was stated by Alcaide et al.[15]

Our study showed high sensitivity and specificity, making it useful as a good tool for diagnosing tuberculosis. The assay could be useful in a country like ours where the disease is highly prevalent and a prompt diagnosis is important from both health and economic points of view. Phage assay is a rapid, reliable and cost-effective method. It does not require specialised techniques and is easy to perform. The test is sensitive enough to detect and confirm clinically suspected smear negative cases. Moreover, since it gives result within 2 days, it hastens the diagnosis of the disease, thereby helping in the treatment of the same. It can also be recommended as an additional diagnostic test in the health centres. However, more research needs to be conducted to determine its usefulness at the peripheral level.

The cost of the test though a little more than sputum microscopy, can still be included as a routine diagnostic procedure since it will cut down hospital visits, hospital stay, morbidity and mortality resulting from Tuberculosis which in turn will further the economic growth of the country.

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