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EVALUATION OF THE USEFULNESS OF PHAGE AMPLIFICATION TECHNOLOGY IN THE DIAGNOSIS OF PATIENTS WITH PAUCIBACILLARY TUBERCULOSIS

*D Biswas, A Deb, P Gupta, R Prasad, KS Negi

Abstract

The present study was undertaken to assess the performance of the Fast Plaque TB™ (FPTB) test in the diagnostically difficult group of paucibacillary tuberculosis (TB) and to compare its results with the conventional bacteriological methods. The study was conducted on a total of 139 patients, who were negative for TB in sputum-smear examination. Bronchoalveolar lavage (BAL) or pleural biopsy specimens collected from these patients were subjected to smear examination, LJ culture and FPTB test. The smear, culture and the FPTB positivity rates were compared between patients with pulmonary and pleuro-pulmonary involvement. The FPTB test was found to register an overall sensitivity of 58.8% and specificity of 97.9%. The positive and negative predictive values of the test were 98.1 and 56.5, respectively. Among patients with paucibacillary TB, on head-to-head comparison, we found that the sensitivity and specificity values of the FPTB test were marginally better than smear-microscopy and inferior to culture on LJ media.

Key words: Paucibacillary tuberculosis, phage amplification assay, tuberculosis

Though tuberculosis has staged a comeback in the developed world owing to the ongoing AIDS pandemic, 95% of the cases and 98% of the deaths still occur in the developing countries.^[1] India, which accounts for a third of the global tuberculosis burden, registers two million new cases every year, which is far more than that in any other country.^[2] The key to combating the menace lies in the early and accurate diagnosis, followed by institution of prompt and appropriate therapy.

The Fast Plaque TB™ (FPTB) test (Biotec Laboratories Ltd, Ipswich, Suffolk, UK) utilizes mycobacteriophages (Actiphage™), to achieve a rapid diagnosis of tuberculosis within 48 hours. Considering the importance of early diagnosis and the technical simplicity of the test, this assay can be used in resource-poor countries with a high disease burden, provided it is found to be adequately sensitive and specific in such settings. Several studies have been carried out to assess the diagnostic utility of the test in various countries.^[3-9] But, no study has yet focussed exclusively on the group of sputum-negative paucibacillary patients, who constitute a sizeable fraction of tuberculosis patients attending a referral hospital of a developing country. Establishment of a definitive diagnosis is often problematic in this group of patients.

The present study was designed with the objective of ascertaining the diagnostic usefulness of this test in

paucibacillary sputum-smear negative patients and to determine its ability to differentiate tuberculosis from diseases that have similar clinical presentations.

Materials and Methods

Study population

The study was conducted on a total of 139 patients attending the Pulmonary Medicine OPD of Himalayan Institute of Medical Sciences, which is a 750-bed tertiary care teaching hospital in India. Consecutive patients were included in the study with the exclusion of the following categories of patients:

- Patients with positive sputum-smear (at least one sputum-smear found AFB positive out of three consecutive specimens).
- Patients who gave history of antitubercular treatment (ATT).
- Patients with pleural effusion, in whom sputum and/or pleural fluid was found to be smear positive for AFB.

The inclusion criteria for the study was clinical suspicion of tuberculosis corroborated with radiological, cytological and/or histopathological findings. The provisional diagnosis of all the patients was confirmed in the course of the study either with the help of microbiological evidences from BAL fluid or pleural biopsy samples or by observing improvement with ATT. On the basis of available clinical data and investigational findings, the study population was divided into the following groups:

- Group I: Patients with sputum-negative Pulmonary TB; diagnosed on the basis of clinical features, suggestive chest X-ray, with or without smear-microscopy by Ziehl Neelsen (ZN) staining and culture on LJ (IUT) medium

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of BAL fluid ($n = 53$). All of these patients were found to be negative for AFB on three consecutive sputum-smear examinations.

- b. Group II: Patients with pleuro-pulmonary disease; diagnosed on the basis of clinical profile, lymphocyte-rich exudate, absence of malignant cells, histopathologic finding of granuloma in pleural biopsy, with or without smear-microscopy and LJ culture of pleural biopsy ($n = 37$).
- c. Group III: Patients with other simulating conditions, like acute exacerbation of COPD, bronchogenic carcinoma, aspergilloma, malignant pleural effusion. In all of these patients, co-existence of tuberculosis was conclusively excluded on the basis of clinical history and examination, radiological examination and LJ culture ($n = 49$).

Sample collection

Bronchoalveolar lavage fluid was collected from patients who had no pleural involvement (Groups I and III) and pleural biopsy was collected from patients having pleural involvement (Group II and patients with malignant pleural effusion in Group III). Informed consent was obtained before the collection of samples. All the samples were collected before the initiation of ATT.

The samples were suitably coded to conceal patient information and were sent to the laboratory for ZN staining, LJ culture, histopathological examination (wherever appropriate) and FPTB test.

Sample processing

All samples were processed within 24 hours of collection. BAL fluid specimens were subjected to the standard N-acetyl-L-cysteine-NaOH digestion-decontamination procedure. The sediment, obtained after neutralization and subsequent centrifugation, was suspended in 1 mL phosphate buffer, pH 6.8. Smears for ZN staining were prepared from this suspension and 0.5 mL of the suspension was inoculated on LJ medium. Pleural biopsy specimens were homogenized, centrifuged and the sediment was used for smear examination and LJ culture. Smears were examined independently by two experienced observers. Inoculated LJ media were incubated at 37 °C and examined at weekly intervals for upto 8 weeks.

Species identification of isolates

Positive LJ slants were initially examined by ZN staining and then subjected to sensitivity test with para-nitro benzoic acid (PNB). The isolates sensitive to PNB were classified as belonging to *Mycobacterium tuberculosis* complex.

FAST plaque TB™ test

The test was carried out and interpreted in accordance with the manufacturer's instructions. After preparation of smears and inoculation of LJ slants, the remaining sediment was suspended in 15 mL of FPTB Medium Plus (Reagent C),

prepared from reagents supplied by the kit manufacturers. It was centrifuged for 20 minutes at 2000×g and the resultant pellet was re-suspended in 1 mL of Reagent C. One millilitre of the suspension was aseptically transferred to a reaction vessel and incubated overnight at 37 °C. Actiphage™ (0.1 mL) was added to each reaction tube and incubated at 37 °C for 1 hour. After incubation, 0.1 mL of Virusol™ solution was added to each sample and mixed thoroughly. After allowing to stand for 5 minutes, 5 mL of reagent C was added to each vessel, followed by 1 mL of sensor™ cells. The entire contents of the vessel were transferred to a sterile petri dish containing 5 mL of molten FPTB Agar. After properly mixing the contents and allowing it to set, the plate was placed in a 37 °C incubator overnight. The next morning, the plates were examined for the number of plaques. Specimens showing 20 or more plaques or confluent lysis of the sensor cells were recorded as positive and those showing 0-19 plaques were considered negative. With each batch of tests, positive and negative controls were also tested using reagents supplied with the kit. The tests were considered valid if the negative control showed less than 10 plaques and the positive control showed more than 20 plaques.

Statistical analysis

The smear, culture and the FPTB positivity rates were compared between patients with pulmonary (Group I) and pleuro-pulmonary involvement (Group II), by applying the Chi-square test. FPTB positivity rates were also determined among smear-positive and culture-positive, smear-negative and culture-positive, smear-positive and culture-negative, and smear-negative and culture-negative groups. Presence of statistically significant difference between the positivity rates in the four groups was ascertained by applying the Chi-square test. Sensitivity, specificity, positive and negative predictive values were determined by standard methods.

Results

Table 1 depicts the results of the three diagnostic tests used in the study, viz., smear-microscopy, LJ culture and FPTB. In all, out of the 90 tubercular patients, 49 (54.4%) were smear-positive and 63 (70%) were culture-positive. All the isolates recovered on LJ culture were found to belong to *M. tuberculosis* complex and no atypical mycobacterium was isolated in the study. Among the 49 non-tubercular patients included in Group III, two were smear-positive and none were culture-positive. Both these patients showed 1-2 bacilli/300 fields. But since the smear-positivity did not correlate with clinical and radiological presentation and LJ culture were negative, these AFB-positive smears were considered to be due to contaminating environmental mycobacteria and hence, treated as false-positives. Fresh samples could not be obtained from these patients for repeat smear examination. The sensitivity and specificity values of smear microscopy were thus found to be 54.4 and

Table I: Results of FPTB test in Groups I and II according to smear and culture results

	Group I (n = 53)				Group II (n = 37)				Total
	LJ +ve (n=43)		LJ -ve (n=10)		LJ +ve (n=20)		LJ -ve (n=17)		
	FPTB +ve	FPTB -ve	FPTB +ve	FPTB -ve	FPTB +ve	FPTB -ve	FPTB +ve	FPTB -ve	
Smear +ve	25	6	-	2	8	3	-	5	49
Smear-ve	9	3	3	5	6	3	2	10	41
Total	34	9	3	7	14	6	2	15	90

95.9%, respectively, while the corresponding values for LJ culture were 70 and 100%. The FPTB test was positive in 53 of the 90 tubercular patients and one of the 49 non-tubercular patients; thus registering an overall sensitivity of 58.8% and specificity of 97.9%. The positive and negative predictive values of the test were found to be 98.1 and 56.5%, respectively. Neither of the tests showed statistically significant difference in the positivity rates between patients with pulmonary and pleuro-pulmonary involvement ($P > 0.05$).

The FPTB assay positivity rate was 78.6% (33 out of 42) among smear- and culture-positive patients, 71.4% (15 out of 21) among smear-negative and culture-positive patients, 0% (0 out of 7) among smear-positive and culture-negative patients, and 25% (5 out of 20) among smear- and culture-negative cases. This difference in the positivity rates between the different groups was found to be statistically significant by the Chi-square test ($P < 0.001$). Among the culture-positive samples, 76.19% (48 out of 63) were FPTB-positive, while 18.52% (5 out of 27) were FPTB-positive among the culture-negative cases.

In head-to-head comparison, the FPTB test performed slightly better than smear-microscopy, while culture was found to be the most sensitive and specific diagnostic modality (Table 2).

Discussion

Apart from the colossal magnitude of the disease, tuberculosis is associated with certain unique diagnostic problems in developing countries like India. Majority of TB patients seek their initial consultation from private practitioners, many of whom rely more on chest X-rays than on sputum microscopy, prescribe non-standard drug regimens, have no means to monitor compliance and do not

maintain any patient record.^[10] Besides, the wide availability of antibiotics over-the-counter enables patients to receive drugs like fluoroquinolones, which have anti-mycobacterial action too. Because of poor literacy rates, often patients are unable to report their drug intake history properly. These factors account for the fact that a referral hospital in a third-world country receives a group of patients in whom diagnosis of TB cannot be arrived at by means of conventional bacteriological methods. Since the present study deals with this diagnostically difficult subset of patients, unlike most other studies, LJ culture was not considered as the gold standard here.

The test showed poorer sensitivity in our study than in earlier studies^[3-9] primarily because in those studies, a majority of the patients included had been sputum-smear positive. It had been demonstrated by Alcaide *et al.*,^[3] that the test is more likely to give positive results for specimens in which high number of acid-fast bacilli were observed in the smear and from which recovery of the bacilli was highest (>30 colonies isolated/slant). The lower sensitivity could also be due to the inclusion of a single specimen from every patient. Though the WHO recommends the examination of multiple consecutive specimens to enhance case detection rates, the present study was limited to testing of single specimens because of the invasive nature of the procedures involved in sampling. To our knowledge, the lone study that had included non-sputum samples^[3] has reported a sensitivity of 58.3%, which is similar to that obtained in our study.

In a recently published meta-analysis on the diagnostic utility of phage-based assays, it was observed that these tests have high specificity (83-100%), but modest and variable sensitivity (21-88%). Analysis of studies that directly compared phage tests with microscopy and culture as a common reference standard suggested that overall accuracy of these tests was slightly better than smear-microscopy.^[11] Our findings on the comparative usefulness of smear-microscopy and FPTB test, showing marginally improved sensitivity and specificity values with the latter, are in broad agreement with the findings of this meta-analysis.

Discrepant results between the diagnostic methods were obtained by us in a number of patients. The results of smear-microscopy and FPTB test were not in agreement

Table 2: Comparison of the three diagnostic methods used in the study

Method of diagnosis	Sensitivity (%)	Specificity (%)
Smear - Microscopy	54.4	95.9
FPTB	58.8	97.9
LJ Culture	70	100

in 36 patients, while LJ culture showed discordant results with FPTB test in 20 patients. Of the smear-negative and culture-positive patients ($n = 21$), 15 were found to be FPTB test-positive. All the six false-negative cases in this category showed delayed growth, between 6 and 8 weeks of incubation. Since the diagnostic threshold of the test has been reported to be 10 cfu of bacilli in a 100- μ L sample,^[3] the negative results could be accounted for by low bacillary counts. This is also the reason for delayed culture-positivity with these specimens (Culture can detect 10-100 organisms/mL of specimen).

Culture-negative patients accounted for 30% of our study population. Seven out of these 27 patients were found to be smear-positive. Though patients were categorically asked about history of receiving ATT, it cannot be ruled out that owing to poor awareness levels and prevalent social stigma, some patients might not have given a correct disclosure of such treatment. Hence, it could be that the bacilli seen in the smears of these patients were dead bacilli, which could not be recovered on culture. Culture-positivity would also have increased if we had used a combination of liquid and solid media or multiple solid media, instead of culturing only on LJ media. Though both culture and FPTB tests rely on the presence of viable bacilli, it is not exactly clear why 5 of the 27 culture-negative patients were FPTB-positive. It could be that these patients were infected with bovine strains of tubercle bacilli and the LJ media being used (glycerol-enhanced) could not support the growth of bovine strains of tubercle bacilli, particularly when present in less numbers.

Among the non-tubercular controls, one patient was found to be positive by the FPTB test; 20 plaques were found from the bronchoscopic specimen of this patient, which was just equal to the cut-off value recommended by the kit manufacturers. Since there was no other corroborative finding in this patient (negative for sputum-smear and culture), he was not put on ATT. Such false-positivity with low plaque numbers could be caused by incomplete destruction of exogenous phages by the virucidal solution, possibly due to a protective effect on the phage by respiratory secretions.

To conclude, the FPTB test did not show significantly improved sensitivity and specificity values, compared to conventional bacteriological methods, in paucibacillary TB. This study focuses on the applicability of the FPTB test exclusively in non-sputum samples, which has not been

studied extensively elsewhere. However, since none of our patients were found infected with atypical mycobacteria, the test needs to be evaluated in this group of patients to obtain a clearer picture of its specificity. The rapidity of the test could be of advantage when compared to LJ culture and the test could be used to monitor response to therapy, because of its reliance on the presence of viable bacilli.

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