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RAPID DIAGNOSIS OF PULMONARY TUBERCULOSIS BY PHAGE ASSAY IN HIV POSITIVE PATIENTS

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ABSTRACT

Tuberculosis is commonest opportunistic infection in HIV positive patients, in India. Rapid progression of tuberculosis in HIV infected patients makes early diagnosis critical. Though smear microscopy remains the mainstay of diagnosis, has low specificity. Culture techniques are sensitive and specific but are slow while molecular methods are rapid but expensive. Phage amplification which utilizes specific mycobacteriophages to detect viable *M.tuberculosis* in respiratory samples is simple and rapid. This study was done to show, comparative evaluation of Phage assay with sputum microscopy and culture on Lowenstein-Jensen media in HIV seropositive patients. Three consecutive sputum samples were collected and subjected to ZN smear. After decontamination and concentration techniques, samples were used for Phage assay, cultured on LJ and smears were prepared. The overall sensitivity, specificity, PPV and NPV of Phage assay when compared to LJ culture was 83.3%,100%,1.0and 0.92 respectively. With respect to smear-negative specimens, the sensitivity was 50%.

KEY WORDS: **Tuberculosis, *M.tuberculosis* , Phage amplification , Lowenstein-Jensen media**

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INTRODUCTION

HIV is the most potent risk factor for the progression to active TB among those infected with TB-HIV; as a result TB is the most common life threatening opportunistic infection associated with HIV and biggest cause of death among patients with acquired immunodeficiency syndrome, although it is a preventable and curable condition[1]. One of the principles of tuberculosis control is rapid and accurate diagnosis to initiate early therapy. The traditional methods for demonstrating tubercle bacillus, the Ziehl-Neelsen acid fast stain lack specificity and sensitivity, while culture on Lowenstein-Jensen medium requires several weeks. The newer rapid diagnostic methods such as BACTEC or molecular techniques are expensive plus require specialist personnel and equipment limiting their use especially in developing countries[2]. In HIV positive patients, TB is often associated with unusual clinical presentation, atypical chest X-ray findings, AFB smear negative disease and rapid progression. Hence there is a need for rapid, sensitive, low cost technique. Phage amplification offers the potential for such testing which utilizes mycobacteriophages to reflect the presence of viable *M.tuberculosis* with in a specimen[3]. The tests are simple, require no specialized equipment. Results are rapid, available within two days and in terms of plaques which are easy to interpret[4]. Bacteriophage infects TB bacilli in the sample. Following infection, the sample is treated with a potential virucide to destroy mycobacteriophages that have not infected host cells. Following this step the virucide is neutralized so that the subsequently released phages are not affected. The only remaining phages are those protected within the host organisms. These phages undergo a replication cycle resulting in the release of phage from host cells. Released phage are allowed to infect a lawn of rapid growing, non-pathogenic mycobacteria within an agar plate, which also support the replication of the bacteriophage. Zones of clearing (plaques) appear in the presence of TB cells that were originally infected, causing successive rounds of replication in the sensor cells leading to localized zones of cell death and clearing of the lawn. The test's manual formats allow it to be performed in any laboratory that has access to basic microbiological equipment, since no specialized, dedicated equipment is required.

MATERIALS AND METHODS

The present study was conducted at the Department of Microbiology, Bangalore Medical College, for a duration of a year. The study group comprised, a total of eighty patients who were above 15 years of age and diagnosed to be HIV positive(according to the NACO guidelines), attending Victoria and Bowring & Lady Curzon Hospital. These patients were selected based on clinical suspicion of pulmonary tuberculosis with symptoms such as cough of more than two weeks, fever lasting two-three weeks, chest pain, hemoptysis, and unexplained weight loss. Patients who were on anti tubercular medication and children below 15yrs of age were excluded from the study. Three sputum samples were collected for AFB Ziehl-Neelsen (ZN) microscopy, culture on Lowenstein-Jensen (LJ) media and phage assay. The samples collected were processed either on the same day or stored at 2-8 °centigrade and processed within 3 days of collection. All the sputum samples were handled carefully in a biosafety cabinet .Smears were done from the sputum received, samples both before and after concentration techniques.

PHAGE ASSAY AND CULTURE

The assay was carried out by using FAST Plaque TB kit (Biotec Laboratories, U.K).All specimens wee processed according to the manufacturer.

Decontamintion of sputum specimens for culture and phage assay.

Samples were processed by N-acetyl –L-cysteine sodium hydroxide method.

Assay procedure

Both positive and negative controls were included in the assay and tested as per the manufacturer's instructions. Negative control contained 1 ml of plain Fast Plaque TB broth; whereas three positive controls were prepared by serial dilution of *M.smegmatis* respectively. For the assay 1ml of decontaminated and concentrated sediment was mixed with 1ml of Fast Plaque TB broth and incubated at 37^{O}c over night to enrich viable TB bacilli present in the sample. After enrichment, 100ul of mycobacteriophage solution was added and incubated for further 1 hour to allow infection to take place. Then 100ul of virucide solution was added for destruction of all bacteriophages which have not infected host cells and incubated at room temperature for 5 minutes. Then 5ml Fast plaque TB medium was added to neutralize excess of virucide, followed by 1ml of helper cells. After mixing thoroughly it was added to the petridish and overlaid with 5ml of molten agar. On pouring, plates were rotated several times, both clockwise and counterclockwise. Plates were allowed to set and they were incubated at 37^{o}c 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.

Culture

Sputum specimens were also subjected to Modified petroff's decontamination and concentration technique and the sediment was inoculated onto a slant of LJ medium. Two slants (one inoculated after NALC_NAOH concentration technique and another after Modified petroff's method) were incubated at 37^{o}c for 8weeks.The slants were inspected for growth everyday for first week and then weekly for seven weeks and the results were recorded. Smears from the positive LJ cultures were prepared and identified by standard techniques.

STATISTICAL ANALYSIS

The statistical analysis was done using the results of the present study. AFB smear and phage assay test results were compared with the culture results as the Gold standard. The Sensitivity, Specificity, Positive predictive value and Negative predictive values of the tests were calculated .Calculations for the derivation of P value were done using Fischer's exact test by accessing the website Graph pad.com.

RESULTS

Out of the 80 HIV seropositive patients, samples from 4 patients showed contamination on LJ media hence were excluded from the analysis of the results. Of the 24 (31.57%)HIV-Tb co-infected patients,75% were males and 25% were females.

Table 1
Comparison of AFB smear with Culture

n=76	Culture positive	Culture negative	Total
AFB Positive	18	4*	22
AFB Negative	6	48	54
Total	24	52	76

p value = <0.0001 (Statistically significant)
**** Positive only by concentrated smears.***

Table 2
Comparison of Phage Assay with AFB Smear

n=76	AFB Positive	AFB Negative	Total
Phage assay Positive	17	3	20
Phage assay Negative	1+4*	51	56
Total	22	54	76

p value = < 0.0001 (Statistically significant)
**** = Samples were positive only by concentrated smears***

Table 3
Comparison of Phage Assay with AFB Smear

n=76	AFB Positive	AFB Negative	Total
Phage assay Positive	17	3	20
Phage assay Negative	1+4*	51	56
Total	22	54	76

p value = < 0.0001 (Statistically significant)
**** = Samples were positive only by concentrated smears***

Table 4
Comparison of Phage Assay with LJ Culture in Smear Negative Specimens

Smear negative specimens(n=54)	Culture positive	Culture negative	Total
Phage assay positive	3	0	3
Phage assay Negative	3	48	51
Total	6	48	54

p value = <0.0001 (Statistically significant)

Table 5
Sensitivity, Specificity, PPV and NPV of Phage Assay

	All specimens	Smear positive specimens	Smear negative specimens
Sensitivity	83.3%	94.4%	50%
Specificity	94.4%	100%	100%
Positive predictive value	1	1	1
Negative predictive value	0.90	0.80	0.94

DISCUSSION

Rapid and accurate diagnosis of TB aids in proper management of the disease. Phage assay is simple, rapid and also a safe technique as the bacilli are rendered non infective by bacteriophages. Our study showed a sensitivity of 77.27% and specificity of 94.44% for phage assay when compared with ZN smear. Out of the 5 smear positive but phage assay negative specimens, 4 specimens were direct ZN smear negative but concentration smears were positive with few AFB. Phage assay negative could be due to the presence of very less number of bacilli in the sample, which could be below the analytical sensitivity of the assay.(Analytical sensitivity of the phage assay is 100-300 bacilli/ml).

Table 6
showing comparison of Phage assay with LJ culture

Authors	Sensitivity	Specificity	Positive predictive value	Negative predictive value
Shennai et al(2002)[4]	93%	88.2%	0.93	0.88
H.Albert et al (2002)[7]	72.5%	99%	0.91	0.96
Ayman Mohamed Marie et al (2003)[2]	76.5%	95%	-	-
Olive Igbuzor et al (2005)[5]	96%	93%	-	-
Meta analysis(2005)[6]	21-88%	83-100%	-	-
Anna Lynda Bellen et al (2003)[8]	31%	86%	0.7	0.55
Katiyar et al [9]	90.7%	96%	97.5%	85.75%

The overall sensitivity, specificity, PPV and NPV of Phage assay when compared to LJ culture were 83.3%,100%,1.0and 0.92 respectively in our study. The results of our study correlates with the other studies as shown in the table above except that Anna Lynda Bellen et al reported a low sensitivity of 31% and specificity of 86%. There is significant variation in the sensitivity data throughout the literature. The heterogeneity in the sensitivity data may be due to many factors that vary from setting to setting and include transport and the potential impact of environmental conditions on specimen viability. However the evaluation of phage assay, only by Olive igbuzor et al was in HIV sero-positive while others studied among HIV sero-negatives. Since Phage assay detect only viable TB bacilli in clinical specimens, the results of the test are unlikely to be compromised in HIV co-infection. In smear and phage assay negative but culture positive specimens, growth took longer than average time (5-6weeks) and were scanty indicating low numbers of bacilli or poor mycobacterial viability which could be the reason for negative Phage assay test. In one of our case, the smear was 1+, culture had growth but was negative by Phage assay. Several explanations have been stated for this Albert H et al has reported that Phage assay requires viable bacilli and intact phage attachment and replication. Expression of phage receptors and efficiency of phage replication may vary depending on the strain of TB or the physiological state of the bacilli resulting in the variation of the phage-TB interaction. Positive phage assay but negative cultures were not observed in our study. Smear microscopy detected 75% of culture confirmed cases in HIV infected patients. Smear microscopy remains the simplest and inexpensive tool for the rapid screening of TB cases even in HIV seropositive patients. Smear microscopy can be performed even at field and primary care settings.

CONCLUSION

Phage assay do not have substantially higher accuracy than smear microscopy but in comparison to other rapid diagnostic techniques, Phage assay is simple to perform, does not require any sophisticated equipments. Results are easy to interpret and are available within 48 hours aiding prompt initiation of therapy. Overall, Phage assay have high specificity but modest sensitivity. However in smear negative cases, the sensitivity is low. Phage assay could be used in conjunction with sputum smear microscopy for rapid detection of additional cases that would otherwise be missed, when only smear microscopy is used.

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