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Diagnostic Role of Cartridge Based Nucleic Acid Amplification Test in Diagnosing Tuberculosis in Patients Co-infected with Human Immunodeficiency Virus

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Abstract
Background: *During recent years, disease caused by human immunodeficiency virus (HIV) and tuberculosis (TB) have been the two leading infectious diseases associated with mortality worldwide. The delays in diagnosis and initiation of anti-tubercular treatment often lead to increased transmission of TB in the community and spread to extrapulmonary sites within the patient. Our study aimed at to assess the role of CBNAAT in early diagnosis of TB in HIV patients and detection of M. tuberculosis in sputum by CBNAAT compared to conventional sputum microscopy in pulmonary TB*
Methods: *The study screened 211 patients with clinical symptoms suggestive of pulmonary tuberculosis. 30 subjects were found to be seropositive for HIV. Out of 30 HIV-TB patients, 20 were male. Direct microscopic sputum examination was done using Light Emitting Diode based Fluorescent Microscopy (LED-FM) method. All patients also submitted sputum sample for GeneXpert MTB/Rif test, a cartridge based nucleic acid amplification test (CBNAAT), analysed using GX4 system.*
Results: *LED-Fluorescent Microscopy sputum examination was positive for acid fast bacilli in 8/30 HIV seropositive patients and 131/181 subjects who were seronegative for HIV. The data suggested patients who were HIV positive were less likely to have sputum smear positive results. CBNAAT detected M. tuberculosis in 17/30 HIV-seropositive patients and in 133/181 HIV-seronegative subjects. 56.7% of the patients who were positive for HIV were also positive for MTB and two of them had rifampicin resistance detected by CBNAAT.*
Conclusions: *CBNAAT significantly increased the conclusive diagnosis of tuberculosis in patients co-infected with HIV, and additionally detected rifampicin resistance, thus providing huge leap in management of tuberculosis in HIV setting.*
Keywords: *Cartridge based nucleic acid amplification test, HIV, Tuberculosis, GeneXpert MTB/Rif test, Rifampicin resistance.*

Introduction

Human immunodeficiency virus (HIV) and tuberculosis (TB), individually, have been among top ten causes of mortality all over the world [1]; both HIV and TB were the two leading causes of infectious disease associated mortality worldwide. People living with HIV are more likely than others to develop tuberculosis. Additionally, TB is the leading cause of death among *People Living with HIV/AIDS* (PLHA). Nearly one in four deaths among people with HIV can be attributed to TB. In 2015, amongst persons who had both TB and HIV, 400,000 are estimated to have died, in addition to the 1.4 million people who died from TB alone.[1]

In most regions where tuberculosis is a considerable problem, diagnosis conventionally relies on microscopy. However, TB microscopy has a sensitivity of only 40–60 % under field conditions, falling to as low as 20 % in the presence of HIV co-infection.[2] In HIV-TB coinfected patients the sputum is often scanty and the numbers of bacilli are not huge in sputum due to rarity of cavitary lesion or caseous necrosis. As a result, sensitivity and specificity of sputum microscopy as a diagnostic tool is decreased. The conventional gold standard test, sputum culture for mycobacterium, is not suitable for screening purposes being time consuming, taking 4-8 weeks, and a reliable testing facility not widely available. The delays in initiation of anti-tubercular treatment often lead to increase in the risk of transmission of TB in the community and spread to extrapulmonary sites within the patient[2]. There is an urgent need to have a diagnostic tool fast enough to provide results within few days and with a high degree of sensitivity and specificity.

Cartridge based nucleic acid amplification test (CBNAAT), specific for *Mycobacterium tuberculosis*, has been recently introduced for detection of TB. It has an added advantage of detecting rifampicin resistance as it targets the rpoB gene of mycobacteria, which is the critical gene associated with rifampicin resistance. The test is highly specific and does not give cross reactions with any other bacterial species including a comprehensive panel of mycobacteria thereby excluding non-tubercular mycobacteria. In a study, the overall sensitivity, specificity, positive predictive value and negative predictive value of CBNAAT test (Gene Xpert MTB/RIF assay) were found to be 98.6%, 100%, 100% and 93.8%, respectively.[3] However, the role of CBNAAT in the diagnosis of TB in HIV patients has not been widely studied. We planned this study to evaluate the role of CBNAAT in early diagnosis of pulmonary TB in HIV patients by detection of *M. tuberculosis* in sputum using CBNAAT compared to conventional sputum microscopy.

Methods

The present study was undertaken at the Departments of Respiratory Medicine and Microbiology at our Institute. All patients attending the Respiratory Medicine Department during study period with symptoms suggestive of tuberculosis (Box-1) and who were diagnosed to be seropositive for *Human Immunodeficiency Virus* detected using ELISA test were further investigated for diagnosis of tuberculosis.

Box 1:
Diagnostic evaluation of patients with any one or more of the following clinical presentation:
Clinical symptoms
Cough more than two weeks
Fever more than two weeks
Hemoptysis
Significant weight loss
Significant decrease in appetite
History of any tuberculosis treatment in past
Chest radiograph with pulmonary lesion suggestive of tuberculosis

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Direct microscopic sputum examination was done at *Designated Microscopic Centre* in the Department of Respiratory Medicine running in collaboration with Revised National Tuberculosis Programme (RNTCP). Two samples of sputum, one sample collected on spot under supervision and other collected early in the morning, were evaluated for presence of *acid*

fast bacilli under Light Emitting Diode based Fluorescent Microscopy (LED-FM)

Cartridge based nucleic acid amplification test:

The Xpert MTB/Rif test is a cartridge-based fully automated NAAT (nucleic acid amplification test) currently recommended by WHO [4] and adopted by Revised National Tuberculosis Control Programme run by Government of India for detection of tuberculosis case and rifampicin resistance. The underlying principle of Xpert assay being detection MTB and RIF resistance by polymerase chain reaction based amplification of the 81-bp *rpoB* gene segment and probing for the mutations that are related to rifampicin resistance. The assay is automated and completes within 2 hours, with minimal hands-on technical time.[5,6] Although molecular amplification is already a proven technology in TB diagnosis, other existing tests are too complex for routine and widespread use in field conditions at peripheral level. GeneXpert, the test device platform, was launched by Cepheid in 2004 and simplifies molecular testing by fully integrating and automating the three processes (sample preparation, amplification and detection) required for real-time PCR-based molecular testing. The Xpert MTB/RIF test uses a cartridge containing all elements necessary for the reaction, including lyophilized reagents, liquid buffers and wash solutions.

A minimum of 2 ml of sputum sample was collected in a falcon tube. The sample was diluted with reagent, incubated at room temperature and then loaded into cartridge and analysed for presence of mycobacteria and rifampicin resistance in GX4 System (with 4 modules).

Statistical Analysis

Statistical analyses were done using SPSS 24 software. The tests used were Pearson chi-square, Continuity correction, Fisher's exact test, Likelihood ratio, Linear-by-linear association.

Results

The present study screened 211 patients with clinical symptoms suggestive of pulmonary tuberculosis. 30 subjects were found to be seropositive for human immunodeficiency virus, making a prevalence of 14.22% among all tubercular cases and these 30 subjects constituted the study population of current study. Out of 30 HIV-TB patients, 20 (66.6%) were males and 10 (33.3%) were females. This male predominance may be accounted by their migration for employment within and outside the state thereby subjecting them to risk behaviour.

As shown in table 1, direct sputum smear examination by LED-Fluorescent Microscopy showed only 8/30 patients with HIV positive status having a positive report for the presence of acid fast bacilli whereas direct sputum smear examination was able to detect acid fast bacilli in 131/181 subjects who were seronegative for HIV. The statistical analysis (table 2) showed strong inverse association between these two parameters; patients who were HIV positive were less likely to have sputum smear positive results.

The outcome of CBNAAT test in HIV seropositive and HIV seronegative subjects is shown in Table 3. 17/30 patients with HIV positive status showed *M. tuberculosis* presence over CBNAAT test whereas 133/181 seronegative for HIV had *M. tuberculosis*. The statistical analysis as shown in Table 4 suggests that over 50% of the patients who were positive for HIV were also positive for MTB detection in sputum samples by CBNAAT and there was an association between these two parameters to some extent. In addition, CBNAAT also diagnosed rifampicin resistance simultaneously in 2 patients providing a big leap in management of tuberculosis. The advantage of CBNAAT over direct sputum smear examination has been graphically shown in Figure-1. There was a substantial gain in diagnosis of tuberculosis.

Table 1: Table showing direct sputum microscopic status in HIV seropositive and seronegative subjects

		Direct sputum microscopy for AFB		Total
		Negative	Positive	
HIV Seropositive Status	Negative	50	131	181
	Positive	22	8	30
Total		72	139	211

Table 2: Statistical tests to assess impact of HIV status over direct sputum smear results

	Value	Df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	23.919[a]	1	<0.001		
Continuity Correction[b]	21.929	1	<0.001		
Likelihood Ratio	22.714	1	<0.001		
Fisher's Exact Test				<0.001	<0.001
Linear-by-Linear Association	23.805	1	<0.001		
N of Valid Cases	211				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 10.24.

b. P value is <0.001 which is highly significant. There is strong inverse association between these two parameters. The study showed that patients who were HIV positive were less likely to have sputum smear positive results.

Table 3: Table showing outcome of CBNAAT test in HIV seropositive and seronegative subjects.

|		Sputum CBNAAT		Total
		MTB not Detected	MTB Detected	
HIV Seropositive Status	Negative	48	133	181
	Positive	13	17	30
Total		61	150	211

Table 4: Statistical tests to assess effectiveness of CBNAAT in HIV seropositive subjects

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	3.540[a]	1	.060		
Continuity Correction[b]	2.769	1	.096		
Likelihood Ratio	3.325	1	.068		
Fisher's Exact Test				.081	.051
Linear-by-Linear Association	3.523	1	.061		
N of Valid Cases	211				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 8.67.

b. P value is 0.06 which is almost near to the value of 0.05. Hence, there is an association between these two parameters to some extent. More than 50% of the patients who were positive for HIV were also positive for MTB detection in sputum samples by CBNAAT.

Figure 1: Graphical comparison of Sputum Direct Smear and Sputum CBNAAT in providing conclusive diagnosis in patients co-infected with HIV
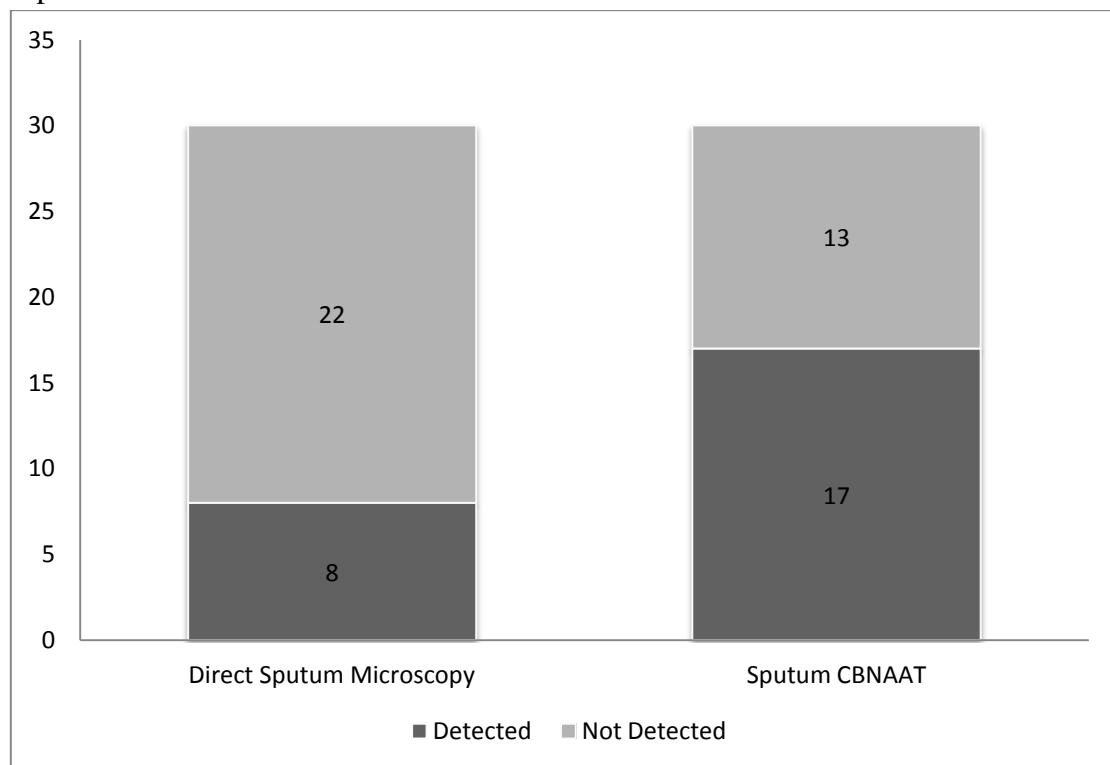
Discussion

Tuberculosis is a major challenge for anti-retroviral therapy services in countries suffering with a high load of HIV co-infection along with a resource-limited socio-economical scenario[7]. These risks are further increased manifold due to increased probability of presence of multi-drug resistant tuberculosis. To address these challenges, there is a critical need for rapid and authentic screening for TB and detection of drug resistance for early initiation of appropriate treatment.

Conventional sputum microscopy, often used as a first line of diagnostic tool due to being a simple, economical, and easy-to-do test, provided diagnosis only in 8 out of 30 subjects in our study. Apart from being an operator dependent test, its sensitivity has been shown to range from 20 % to 60 % under different conditions as it needs over 10,000 bacilli per ml to give a positive result. This sensitivity is further decreased in the presence of HIV co-infection due to rarity of cavitary lesion and sputum production; this often leads to a delayed diagnosis and in worst case scenario, missed diagnoses further leading to increased morbidity and mortality rates and continued spread of TB to contacts[8]. Cattamanchi et al observed the sensitivity of sputum microscopy in HIV co-infection setting to ranges from 43 to 51 percent[9]. Matee et al reported efficacy of microscopy in only 55% of patients[10]. These (false) smear-negative cases are invariably treated with multiple courses of broad-spectrum antibiotics before being re-assessed for TB, only if significant symptoms persisted. Many times, these patients did not come back for a re-assessment or actually felt better with the initial treatment. Delay in treatment of tuberculosis in PLHIV is usually associated with higher mortality[11].

In our study, cartridge based nucleic acid amplification test (CBNAAT) detected 17/30 patients with clinically suspected pulmonary tuberculosis. Additionally, rifampicin resistance was detected in 2 patients. The Xpert MTB/RIF test exhibits high sensitivity and specificity for detecting pulmonary TB disease. An in-vitro study demonstrated a limit of detection of as few as 131 colony-forming units/mL of MTB, compared with approximately 10,000 colony-forming units/mL with conventional smear microscopy. The sensitivity of the MTB/RIF test on single sputum sample has been observed to be 92.2% for culture-positive TB; 98.2% for smear-positive and culture-positive cases; and 72.5% for smear-negative, culture-positive cases, with a specificity of 99.2%[12]. Sensitivity and higher specificity have been higher when three sputum samples were tested. CBNAAT is a useful surrogate test for screening for MDR-TB as past studies on drug resistance have shown that rifampicin resistance seldom occurs alone and around 90 % of rifampicin resistant patients are diagnosed to have MDR-TB[13]. This is having unmatched significance in TB endemic areas like India where there is high prevalence of MDR-TB, around 3% in new cases and 12 to 18% in previously treated cases[13].

Interestingly, in HIV-TB co-infection setting the sensitivity of sputum microscopy has been observed to be substantially decreased, but it does not significantly affect CBNAAT outcome[14]. Studies from high HIV endemicity areas in Peru have also shown that HIV status does not affect the performance of CBNAAT[15]. When combined, smear microscopy and CBNAAT had a significantly better sensitivity than smear microscopy alone in patients infected with HIV having a CD4 count less than 200 cells/ml[8].

A study done in Hyderabad showed incremental case detection of 10.8% when CBNAAT was used to diagnose tuberculosis over and above fluorescent microscopy[16], though, HIV status of the patients was not evaluated in this study. A multicentre assessment carried out at five sites including Peru, Azerbaijan, Cape Town, Durban and India demonstrated sensitivity of CBNAAT

nearing 100 percent[17]. Impact study on CBNAAT under RNTCP found additional 2,493 patients diagnosed with pulmonary TB using CBNAAT compared to sputum microscopy in 2012 among more than 30,000 TB suspects[18]. Thus, CBNAAT was found to have highly significant advantage over microscopy alone.

The WHO policy statement on the use of CBNAAT is available since December, 2010. The initial recommendations were that it should be used as the primary diagnostic test in individuals at risk of having MDR-TB or HIV-associated TB (strong recommendation), and that it could be used as a follow-on test to microscopy in settings where MDR and/ or HIV is of lesser concern, especially in smear-negative specimens (this was a conditional recommendation, recognising major resource implications)[19]. This recommendation applied to the use of CBNAAT in sputum specimens only, as data on its performance (sensitivity and specificity) for testing of extrapulmonary specimens at that time were limited[19]. RNTCP, India adopted CBNAAT from April 2012 with launch as a pilot project in Maharashtra and by the year end, under EXPANDx-TB project, 12 CBNAAT labs were established all over India across different states[13]. Since then, CBNAAT has seen rapid expansion and now widely available as a diagnostic tool.

To conclude, CBNAAT was able to diagnose pulmonary TB in patients co-infected with HIV having greater efficacy than sputum microscopy with the advantage of a rapid diagnosis within 2 hours. It had the additional advantage of detecting rifampicin resistance; to enable to start MDR therapy early.

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