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ORIGINAL PAPER

Detection of pulmonary tuberculosis using cartridge based nucleic acid amplification test (CBNAAT) and fluorescent microscopy

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ABSTRACT

Background*: With the advent of advanced laboratory methods for diagnosis of Pulmonary Tuberculosis (PTB), its detection now is increasingly relied upon rapid diagnostic methods like Cartridge based nucleic acid amplification test (CBNAAT) and Fluorescent Microscopy (FM). No study has been published regarding the effectiveness of CBNAAT and FM. This study aims to better the understanding of these methods for detection of PTB. The objective of the study was to compare CBNAAT and FM for detection of Mycobacterium tuberculosis (MTB) using sputum samples.* ***Methods****: A cross-sectional study was performed among 200 study population. 3 sputum samples from each patient were subjected to CBNAAT and 2 slide smears were prepared for Aura mine- O stained FM. Statistical analysis was done using SPSS 23 by Chi square test. P value less than 0.05 was taken as significant difference for this study.* ***Result****: Thirty-three (17%) patients were positive by FM and 58(29%) by CBNAAT for MTB. Statistical analysis was done and the difference in positive yield was highly significant with P value <0.0001. Six (10%) patients were detected as Rifampicin resistance by CBNAAT out of which 2 were missed by FM. Among HIV patients 7(17%) were detected for MTB by CBNAAT and 1(2.5%) by FM.* ***Conclusion****: CBNAAT is a better method of detection as compared to FM in diagnosis of PTB.*

Keywords*: Gene Xpert MTB/RIF Cepheid, MDR-TB, Fluorochrome stain, LED fluorescent microscope*

INTRODUCTION

India has the highest burden of Tuberculosis (TB) with an estimated incidence of 2.2 million cases out of global incidence of 9 million.[1] There arises a need for rapid and more accurate diagnostic test for TB for prompt treatment and global TB control. In December 2010, World Health Organization (WHO) has endorsed Gene Xpert® MTB/RIF, a Cartridge based nucleic acid amplification test (CBNAAT) which can diagnose active TB disease and multidrug-resistant (MDR) TB in less than 2 hour.[2] Gene Expert is a semi-quantitative nested real-time PCR in-vitro diagnostic test with two uses: (1) The detection of Mycobacterium tuberculosis DNA in sputum samples or concentrated sediments prepared from induced or expectorated sputum that are either acid-fast bacilli (AFB) smear positive or negative. (2) The detection of Rifampicin resistance associated mutations of the rob gene in samples from patients of Rifampicin resistance.[3] An alternative technique to Ziehl-Neelsen (ZN) smear microscopy is Fluorescent microscopy (FM), which is 8-10 % more sensitive than ZN smear microscopy and because AFB can be seen at lower magnification (40x), FM smears can be examined in a fraction (about 25%) of the time needed for ZN smears.[4-6] In 2010, the WHO recommended that LED FM be phased into replace ZN microscopy for TB diagnosis.[7, 8] This study will be the first publication from our institute after installation of CBNAAT. No such similar study has been published before and hence the aim of this study is to better the understanding of CBNAAT and FM as a diagnostic tool for detection of Pulmonary Tuberculosis. The objective is to compare CBNAAT (Gene Xpert® MTB/RIF) with Fluorescence microscopy in detection of Pulmonary Tuberculosis using sputum samples.

METHODS

It was a cross sectional study in the Department of Microbiology, Jawaharlal Nehru Institute of Medical sciences, Imphal, Manipur

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from December 2016 to April 2017. Institutional Ethics Committee (IEC) approval was taken for the study and written informed consent was obtained from all participants for use of their sputum for TB diagnostics research.

Study subjects included patients with clinical suspicion of Pulmonary Tuberculosis including symptoms of Cough with or without expectoration for >2 weeks with evening rise of temperature and /or weight loss, fatigue, haemoptysis, loss of appetite. Patients referred from ICTC (Integrated counselling and testing centre) for detection of TB in PL HIV (people living with HIV/AIDS) and patients with prior history of TB and who are categorized as category 2 in DOTS programme of RNTCP were included. Patients who are 18 years of age were excluded from the study. Three sputum samples were collected from all 200 study patients (one spot sample, one morning sample in sterile disposable wide-mouth containers and another spot sample in falcon tube) for analysis. From the two wide-mouth containers, sputum smears were made on two slides and was stained with special stain (AURAMINE O). It was observed under fluorescent microscope using 40x objective lens for two minutes.[9] The acid fast bacilli (AFB) appeared as bright yellowish orange objects against a dark back ground and positive slides are reported as per RNTCP grading (Table 1).[10]

Table 1 RNTCP Auramine-O fluorescent staining grading

GRADE	LED Fluorescence based sputum smear microscopy (400X Magnification: 1length = 40 fields = 200 HPF)
Negative	Zero AFB/1 length
Scanty	1–19 AFB/1 length
1+ grade	20–199 AFB/1 length
2+ grade	5–50 AFB/1 field on Average
3+ grade	>50 AFB/1 field on Average

The sputum samples from the falcon tube were treated with a sodium hydroxide and is opropanol containing sample reagent (SR). The SR was added to the samples at 2:1 ratio for raw sputum samples and incubated for 15 min at room temperature.[11] The treated samples were then manually transferred to the single-use plastic cartridges with multiple chambers that were preloaded with liquid buffers and lyophilized reagent beads and loaded into the Gene Xpert instrument. Remaining steps were fully automated. The cartridge incorporates a syringe drive, a rotary drive and a filter upon which Mycobacterium tuberculosis bacilli were deposited after being liberated from the clinical material. The test platform employs a sonic horn that inserts into the cartridge base to cause ultrasonic lists of the bacilli and release of the genetic material. The assay then amplifies a 192 bp segment of the rob gene using a hemi nested RT-PCR reaction. CBNAAT and MDR positive will be based on the operational definitions.[12] The standard user interface indicates the presence or absence of M. Tuberculosis and the presence or absence of rifampicin resistance, and a semi-quantitative estimate of the concentration of bacilli as defined by the Cycle Threshold (CT) range (high, <16;medium, 16–22; low, 22–28; very low, >28).[13]

Statistical analysis was done using SPSS 23 by Chi square test. P value less than 0.05 was taken as significant difference for this study.

RESULT

In our study both the majority (40%) of the participants and the positivity for MTB (48%) fall under 41-60 years age range. Of the 200 sputum samples, 33(17%) were positive and 167(84%) were negative for MTB by FM. On the contrary 58(29%) sputum samples were detected by CBNAAT. Additionally, 6(10%) positive samples detected by CBNAAT were also detected with Rifampicin (RIF) resistance.

Table 2 shows the comparison between CBNAAT (Gene Xpert) and FM. On statistical analysis, the Chi square value was 84.766 with 1 degree of freedom. Hence the difference in positive yield between CBNAAT and FM was found to be highly significant with P value <0.0001.

Table 2 Comparison of cbnaat with fluorescent microscopy

FLUORESCENT	CBNAAT		Total
	Mtb detected	MTB not detected	
Detected	32	1	Detected
Not detected	26	141	Not detected
Total	58	142	Total

Table 3 Shows the correlation between CT range of Gene Xpert and RNTCP grading for Fluorescent smear microscopy. Out of 58 samples found to be positive for MTB, 26(48%) samples were missed by FM. Rest 32(55%) were detected by both methods. Only 1(0.02%) was missed by CBNAAT which was detectable by FM as 3+ grading.

Table 3 Comparisons between cycle threshold (ct) range of cbnaat and grading of fluorescent microscopy

Fluorescent	CBNAAT				
	Not detected	Low	Medium	High	Total
Scanty	0	0	4	0	4
1+	0	1	3	6	10
2+	0	0	1	4	5
3+	1	0	6	7	14
Not detected	0	15	10	1	26
Total	1	16	24	18	59

Out of the 40 HIV positive patients, 7(17.5%) were detected to be infected with MTB. All of them were detected by CBNAAT mostly under low category of CT value (22-28). Only 1(2.5%) was detected by FM under scanty grading.

Out of 27 DOTS category II TB patients (treatment failure or relapse or irregular anti-tubercular drugs intake), 14(52%) patients were detected by CBNAAT and 11(41%) were detected by FM. From the total of 6 MDR positive patients, 5 were of DOT category II patients. 3 patients were on irregular ATT and 2 with treatment failure. 2 sputum samples were missed by FM which was later

detected by CBNAAT as MDR-TB under medium category of CT value.

DISCUSSION

Tuberculosis was more common among the younger age group of 25-35 years. But recently the trend has shifted towards the older age group. The 2010 Global Burden of Disease estimates show that 57% of all tuberculosis deaths globally occurred among people older than 50, with more than half of these deaths in those aged 65 and above.[14] In our study maximum positivity for MTB was found in age group 41-60 years age range. This may be ascribed to increased life expectancy and waning immunity in adults. In this study CBNAAT could give a maximum positivity of 29% as compared to 17% of FM. And among these 26 positive samples that were missed by FM, maximum (15 samples) falls in low category of CT value (22-28). This low category signifies lower numbers of MTB bacilli or paucibacillary in these sputum samples. Hence CBNAAT could diagnose PTB in older adults who have sputum smear negative pulmonary tuberculosis that were harder to diagnose and treat than conventional pulmonary tuberculosis.

The fact that FM has difficulty in detecting paucibacillary can be attributed to the fact that only a loop-full of sputum sample was taken to make the smear slides for FM. While 2ml sputum sample for CBNAAT was used for the process. This attribution can be easily avoided with the process of concentration and decontamination of sputum. But it was not done in this study as we were following the RNTCP grading for FM. In a study by Navinchandra, he found out that the sodium hypochlorite concentration technique leads to 44.11% increase in detection of new cases as compare to routine RNTCP method. He even suggested the use of this process in smear negative sputum samples if not all the samples.[15] MTB detection by FM could have been even higher if we also employed a decontamination and concentration method in our study. We propose adoption of decontamination and concentration method by RNTCP for FM and devise a grading system for the same.

In our study 10% of the sputum sample was detected with rifampicin resistance by CBNAAT. This finding was similar with others reported from Punjab (9.9%), Jaipur (11.09%), and Lucknow (27%).[16] For early detection of Rifampicin resistance this assay is useful and hence early intervention can be done. CBNAAT was a better diagnostic test when it comes to diagnosing DOTS Cat II patients. FM could not detect 3 Cat II patients, out of which 2 were MDR strain. And hence it is of utmost importance that Cat II patients be confirmed with CBNAAT.

As our state is one of the six highest HIV prevalent states in India and TB being endemic here, HIV-TB co-infections are expected to rise. It is mandatory to test for TB infection in a HIV positive patient before the initiation of antiretroviral therapy (ART).[17] In this study, 7 patients with HIV infection were detected with TB. All of them were detected by CBNAAT mostly under low category of CT value (22-28). Only 1 was detected by FM under scanty grading. This could be explained as the atypical presentation of HIV and pulmonary TB co-infected patients, who presents with non cavitary lesions of the lungs giving rise to paucibacillary sputum samples.[8]

Even though FM can be reported within 30 min time, it is limited to skilled technical expertise at every step from smear preparation and staining to interpretation of the stained slides under FM. There are more chances of false positive result in FM due to the artefacts which are interpreted at the subjective level. On the other hand, CBNAAT does not required skilled technician or the extensive set up in the laboratory for the prevention of amplicon contamination and does not have any pipetting error so there is less chance of false positive results.

Since MTB culture (which is the gold standard for diagnosis of TB) was not included in the study, the sensitivity and specificity of each test was not determined. One sample which was not detected by CBNAAT was found to be positive with 3+grading in FM. This may be attributed to Non Tubercular Mycobacterium (NTM). In a study by Paul W. Wright, FM cannot differentiate between NTM and MTB which he confirmed using culture.[18] It was not confirmed as culture and identification was beyond the scope of this study. Further study is contemplated using culture and identification.

CONCLUSION

In comparison to FM, CBNAAT is more useful in detection of Pulmonary Tuberculosis as it helps in detection of paucibacillary MTB. An additional feature of rifampicin resistance can also be detected by CBNAAT. This study highlights the importance of setting up CBNAAT at every district level of health care centre and all medical institutes for early and accurate detection and prompt treatment of TB.

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