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CARTRIDGE BASED NUCLEIC ACID AMPLIFICATION TEST : A GAME CHANGER FOR DIAGNOSIS OF PULMONARY TB IN HIV PATIENTS ?

KEY WORDS:

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ABSTRACT

Background: World Health Organization (WHO) has recommended CBNAAT (Cartridge Based Nucleic Acid Amplification test) as first line diagnostic modality for detection of pulmonary TB(Tuberculosis) in HIV patients due to increased case finding rate in HIV patients and rapid diagnosis of MDR-TB(Multi-Drug Resistant TB)

Aim: Our Aim was to compare diagnostic accuracy of sputum microscopy with CBNAAT for detection of pulmonary Tuberculosis in HIV patients

Material and Methods. We screened all patients present to HIV clinic for pulmonary TB.. We collected two sputum samples for microscopy using ZN stain , one sputum sample for CBNAAT and one sputum sample for BACTEC based culture in each patient . Using BACTEC culture positive as gold standard, we determined comparative sensitivity, specificity, Positive Predictive Value (PPV),Negative Predictive value(NPV), Positive Likelihood Ratio (PLR) and Negative Likelihood Ratio(NLR) of CBNAAT and sputum microscopy using SPSS 15.0 software .

Results: 204 patients were included in study .71/204 (34.8 %) patients were sputum culture positive. Overall sensitivity, specificity, PLR, NLR,PPV,NPV of CBNAAT was [85.92(75.6 - 93.0), 97.74(93.5 - 99.5), 38.09, 0.14, 95.3 %, 92.9 %] and of smear microscopy was [39.44 (28.0 - 51.7), 96.99 (92.5 – 99)13.11, 0.62,75%,87.5%] respectively .Ten out of 61 (16.39 %) CBNAAT positive patients were Rifampicin resistant. The Test accuracy as represented by AUROC (Area under receiver operator characteristics) was significantly higher for CBNAAT compared to Sputum microscopy [0.91 versus 0.68 ,p<0.001).

Conclusion: CBNAAT-TB is more accurate, rapidly performed ,valid and reliable alternative to sputum microscopy for detection of pulmonary tuberculosis in HIV patients.

INTRODUCTION.

Tuberculosis is among the most common and deadly opportunistic infections in HIV patients in India.[1] An HIV positive Individual is ten times more likely to develop TB than a non-HIV infected individual. HIV positive patients infected with Mycobacterium tuberculosis show fewer cavitations and hence have lesser bacillary load in their sputum. Thus, detection rate of Pulmonary TB is limited in HIV patients by poor sensitivity of sputum microscopy and poor specificity of symptom screens, chest X ray and clinical diagnostic algorithms in these patients.[2]

Many studies have showed benefit of rapid TB detection and starting treatment in HIV patients. In past two decades ,new approaches like fluorescent microscopy and commercial Nucleic acid Amplification tests(NAAT) have been tried for rapid diagnosis of pulmonary TB . [3] Various antigenic targets on M.TB were tried for diagnosis of TB like 16srRNA and IS6110.[4]However commercial NAAT assays were limited by poor specificity, big instruments and resource intensive set up. . Sensitivity (also called the true positive rate) measures the proportion of positives that are correctly identified as such. Specificity (also called the true negative rate) measures the proportion of negatives that are correctly identified as such . However a systematic review of NAAT for diagnosis of pulmonary TB, revealed a variable sensitivity and specificity due to varying assay characteristics, antigenic targets and laboratory techniques. [2] Thus there arose a need for highly sensitive and specific NAAT assay which was cheaper, reproducible, more specific and less resource intensive. These second generation assays then could be used effectively for diagnosis of Tuberculosis in developing countries with limited infrastructure and resource crunch.

A new second generation real time PCR test called, CBNAAT –TB (Cartridge based nucleic acid amplification technique) was developed and optimised in last decade offering significant improvement over other commercially available Nucleic acid assays in terms of diagnostic accuracy.[3,4,5,6,7,8]. CBNAAT automates the entire process of DNA extraction, amplification and detection in small cartridge with little risk of contamination or aerosol generation**.** CBNAAT-TB uses heminested real time PCR assay to amplify a MTB specific 81 bp sequence of rpoB gene which is also probed with five overlapping molecular beacons for detection of rifampicin resistance. [9,10,11,12]

In 2010 WHO recommended CBNAAT-TB as a first line test for diagnosis of TB in HIV patient,based on various studies validating its excellent diagnostic accuracy in different parts of world. [13-20] CBNAAT-TB can not only diagnose M TB with high sensitivity and specificity but also detect rifampicin resistance rapidly within a span of two hours. Further, Rifampicin resistance has been shown to be a surrogate for MDR-TB.[16] Thus, a CBNAAT positive but Rifampicin Resistant individual is likely to have MDR-TB as well.It has safely been used at sub district level centres across the world with excellent results. [14-20] The training can be imparted within 2-3 days to any health worker with no previous knowledge of molecular genetics or computers. It requires almost 100-150/ml of AFB bacilli for positivity as 10000/ml bacillary load required for sputum microscopy positivity. Though Mycobacterial culture requires a low bacillary load of 10-1000 bacilli/ml and is treated as gold standard; it takes longer time to positivity (2-6 weeks), poses significant aerosol generation risks and requires trained professionals.[14] Due to rapid result(<2 hr), unambiguous read out and little need for training health workers: CBNAAT has rapidly emerged as viable alternative to Mycobacterial culture. At present it has been implemented in 20 centres across India as an Revised National TB Control Program(RNTCP) project where it is done for free. Its cost to the government has been brought down to Rs. 700-800 per test as a result of its mass use. [21]

Our aim was to evaluate comparative diagnostic accuracy of CBNAAT with other diagnostic modalities like smear microscopy, chest x-ray and symptom screens.

MATERIALS AND METHODS :

The study was carried out in HIV clinic and Sputum collection

centre of Rajendra Institute of Medical Sciences .. An HIV ELISA based test was performed in all study participants giving consent with proper counseling. If the test result was positive, a CD4 count was also determined. All HIV positive patients presenting to HIV clinic who were not on any ATT (Anti tubercular therapy) were screened for pulmonary TB and included in the study. The study was cleared by Institutional ethical committee and informed consent was taken from all study participants.

A relevant history of Cough ,night sweats, fever ,weight loss ,haemoptysis and episode of previous tuberculosis ,was taken and physical examination and chest x ray was done in all patients. All patients were enrolled consecutively. Patient was asked to submit four sputum samples .A raw sputum samples was sent for performing CB-NAAT-TB to Specialised Tuberculosis diagnostic lab at Sadar Hospital, Ranchi . Three sputum sample were concentrated ; Two were sent for Sputum microscopy by ZN stain at DOTS Centre , while one was sent for and sputum culture of M.TB by using MGIT(Mycobacterium growth in tube) and read on BACTEC-960 at Department of Microbiology respectively. The standard procedures for CBNAAT, Culture and microscopy were performed on all the patient specimens as laid out in guidelines [22,23] The results of CBNAAT-TB, Sputum microscopy and Mycobacterial culture were interpreted separately by trained technicians blinded to the results of each other. Similarly, Rifampicin sensitivity testing on culture was interpreted without knowledge of results of CBNAAT-TB.

Performance of test
CBNAAT-TB test was done using a 4 cartridge module (gene Xpert Cepheid Inc. Technology) by trained health workers at District Hospital. [23] Three laboratory technicians were trained for four days and obtained proficiency testing after ten runs per person. Sample reagent was added in a 2:1 ratio to untreated sputum The closed sputum container was manually agitated twice during a 15-minute period at room temperature before 2 ml of the inactivated material was transferred to the test cartridge . Four Cartridges were inserted into a four cartridge module machine. After two hours electronic test results were generated on the computer and sent directly from the MTB/RIF test system to the central database. The assay generated results within 2 hours with only fifteen minutes of hands on time.

M. tuberculosis was identified when at least two of the five overlapping molecular beacons which were complementary to 81 bp rpo B gene probes gave positive signals with a cycle threshold (C_T) of ≤38 cycles and that differed by no more than a prespecified number of cycles. The *B. globigii* was used as internal control. The user interface indicated the presence or absence of *M. tuberculosis* and a semi-quantitative estimate of the of bacilli as defined by the C_T range (very low: >28 low :22–28; medium :16–21 high, ≤ 15). When performed on unprocessed sputum samples, the assay can generate results within 2 h with less than 15 min of hands-on time.Tthe difference between the first (early C_T) and the last (late C_T) *M. tuberculosis*-specific beacon (ΔC_T) was observed. Rifampicin resistance was reported when ΔC_T was >3.5 cycles and Rifampicin sensitivity if ≤3.5 cycles

The sputum samples of CBNAAT-TB positive but Rifampicin Resistant pattern on CB-NAAT were sent to an approved lab for detection of MDR-TB (Multi Drug Resistant TB) based on Line Probe Assay(LPA) and Solid Culture technique .

Interpretation of Test
Patients were classified as bacteriologically confirmed (culture positive) TB, clinical TB (culture negative patients with clinical features suggesting TB and responding to ATT), no TB and indeterminate (invalid CBNAAT and contaminated culture).All indeterminate tests were excluded from final analysis.

Statistical analysis
A 2x 2 table was constructed and ROC (Receiver operator characteristic curves) were constructed.

The sensitivity, specificity,Positive and negative predictive values

and likelihood ratios (PPV,NPV,PLR,NLR) and area under ROC (AUROC) of sputum microscopy, CB NAAT-TB, Chest X ray, chronic cough, WHO symptom screen (any symptom out of weight loss, fever, current cough and night sweats) [24] and a clinical algorithm [25] consisting of combination of symptoms and chest x ray were determined against the bacteriologically confirmed culture positive gold standard using SPSS 15.0.

RESULTS :
Out of 250 patients, screened in HIV clinic , 30 patients did not meet inclusion criteria(18 could not generate sputum samples, 8 already on ATT (Anti- tubercular therapy, 4 HIV negative), while 16 were not eligible (10 had contaminated culture,4 had invalid CBNAAT, 2 patients did not give 4 sputum samples). Thus in final analysis 204 patients were included out which 71 were culture positive and 133 were culture negative. There was discordance between results of CBNAAT and culture in thirteen cases. The patient flow is depicted in **figure 1**

The demographic profile of the patients is summarized in **Table 1.**

Performance of CBNAAT/RIF
On using culture positive TB as gold standard, the overall performance of CBNAAT was as follows: sensitivity 85.92(75.6 - 93.0), specificity 97.74(93.5 - 99.5), PLR 38.09, NLR 0.14, PPV 95.3 %, NPV 92.9 %, AUROC 0.918 . The performance of Smear Microscopy were as follows: Sensitivity 39.44 (28.0 - 51.7), specificity 96.99 (92.5 - 99.2),PLR 13.11, NLR 0.62. CBNAAT was significant superior to sputum microscopy with higher sensitivity, specificity and AUROC 0.236 (0.148 to 0.325, p <0.001). **(Figure 2)** Only 1 test of CBNAAT was performed as opposed to 2 sputum smears due to financial constraints CBNAAT. Out of 43 smear negative culture positive cases, CBNAAT correctly picked up 33 cases, thus having sensitivity of 76.74 % in these cases.

When an extended TB definition (including both culture positive and culture negative clinical TB) was taken as reference standard then there was a fall in sensitivity of CNAAT and smear microscopy while their specificity increased implying that these cases of Clinical TB might have been missed by culture due to non viable bacteria, but were detected by smear microscopy and CBNAAT In this group: sensitivity, specificity, PLR ,NLR,PPV and NPV of CBNAAT was [75.00(64.4 - 83.8), 99.17(95.4 - 99.9), 90.00, 0.25, 98.4 % , 85.0 %] while that of smear microscopy was [36.90(26.6 – 48), 99.17(95.4 - 99.9), 44.29, 0.64, 96.9%,69.2%] [**Figure 3 a**.] When CBNAAT was used as an additional strategy to a sputum negative microscopy, the sensitivity of diagnostic algorithm rose to 88.73 (79.5-95), while specificity fell to 94.74(89.5-97.8) [**figure 3 b**]

Rifampicin Resistance and MDR
Ten Out of 61 [16.39% (9 - 27.16)] CBNAAT positive cases came out to be Rifampicin resistant as indicated by signal delay exceeding a ΔC_T value (>3.5), between the earliest and latest cycle threshold values [26] **.** All these Rifampicin resistant cases turned out to be resistant to both Rifampicin and INH , thus being true MDR cases. All the MDR TB cases were sent to a specialised MDR –TB unit for treatment.

Indeterminate tests
4 out of 220 (1.8%) eligible patient had invalid CBNAAT (1 bad sample processing , 2 RT- PCR inhibited, 1 susceptible power supply) and were excluded from analysis .Ten patients (4.5%) had contaminated culture and were excluded from analysis.

Discordant cases analysis
10 patients were CBNAAT negative but culture positive (False negative). All these patients had significantly lower CD 4 count (75.5 ± 42 .77 versus 184.26 ± 77.4, p value <0.001) and lower smear positivity (2/10(20%) versus 24/45(53%) , p=0.11) than CBNAAT positive culture positive group (True positives). Three cases were CBNAAT positive but culture negative (False positive). However two out of these cases had clinical evidence of TB and they responded well to ATT. Two cases were negative on CBNAAT but positive on smear microscopy. One of these showed rapid growth of mycobacteria and resistance to Para nitro benzoic acid and was classified as NTM . (Non- tuberculous Mycobacteria).

Relation of test with Immunosupression

Prevalence of bacteriologically confirmed, culture positive Tuberculosis was higher in AIDS group (CD4 <200) than non- AIDS group.[51/91(56.04%) versus 20/ 113(17.6%). Difference = 38.42%, 95% CI :26.06 to 50.8, P < 0.0001] . Rate of smear positivity (13/51 (25.5%)versus 15/20(75%), Difference = 49.5% ,95% C.I. 27.1%- 71.9%, p=0.0004] was significant lower in pulmonary TB associated with AIDS. All CBNAAT negative culture positive test had lower rates of smear positivity and lower CD4 counts than CBNAAT positive culture positive group . Patient who were on HAART for longer time were more likely to have higher CD4 counts and hence higher rates of smear positivity.

Comparison with other symptoms and diagnostic algorithms

The comparative sensitivity and specificity of chronic cough, WHO symptom screen, abnormal chest X-ray, CBNAAT and smear microscopy is described in Table 2. Chronic cough had relatively lower sensitivity in diagnosis of TB, while any symptom from WHO symptom screen had relatively higher sensitivity but low specificity. Two- third of the patients (45/71) with normal Chest x ray had TB, while one fifth of the patient with abnormal chest X-ray (26/133) did not have TB. Sputum microscopy was very specific for picking TB cases , however it had poor sensitivity in picking up TB cases in HIV patients. On combining with chest X ray and WHO symptom screen, its sensitivity increased though at the cost of specificity.

DISCUSSION:

Cartridge base nucleic acid amplification test is a second generation Real time PCR technique which automates the entire process of DNA extraction, amplification and detection in a small cartridge. Thus it enhances specificity of PCR process as it decreases the risk of cross contamination. Due to its small size and portability , it has been used to significantly increase the yield of pulmonary TB detection in HIV patients in various studies across the world and has been recently endorsed by WHO for the same.[13-20]

We found that CBNAAT-TB has high sensitivity and specificity for detection of pulmonary TB in HIV patients and detected almost eighty percent of cases correctly. Sputum microscopy detected only forty percent cases (43 out of 71) of pulmonary TB , while CBNAAT accurately picked up 76.74 % (33 out of these 43 sputum negative culture positive) of the cases missed by sputum microscopy. Commonly used clinical screens like chronic cough(>2 weeks) picked up only half of the cases , newly approved WHO symptom screen (any symptom out of weight loss, fever, current cough and night sweats) had eighty percent sensitivity but ended up over diagnosing half of the cases. A newly approved clinical algorithm[25] combining WHO symptom screen and chest X-ray had only almost ninety percent sensitivity for detecting TB, but very poor specificity. In view of significant limitations of all these commonly applied strategies for diagnosis of pulmonary TB-HIV co infection, a single sputum CBNAAT assay emerged significantly superior to all methods. (Table 2)

The performance of CBNAAT in our study is similar to its performance in various studies across the world implying that the assay performs well in standardized conditions. The variable sensitivity (75-95%) of CBNAAT in various studies by Theron et al [17] and Lawn etal [16] in Africa, is attributed to variable sputum bacillary load of patient population, pre selection of patients based on symptoms and freezing of sputum used in those studies. We did not preselect patients and all patients referred to HIV clinic were screened for TB, also the sample was analyzed the same day for CBNAAT. These reasons explain excellent test characteristics of this assay in our study. CBNAAT outperformed sputum microscopy and chest X-ray detecting almost twice more cases and 1.5 time more cases respectively. Though newly developed WHO symptom screens and clinical diagnostic algorithm had equivalent sensitivity to CBNAAT, they ended up over diagnosing fifty per cent of cases which may result in over treatment. For this reason, WHO has

recommended using CBNAAT as first line test for diagnosing pulmonary TB in HIV patients. CBNAAT had excellent specificity for diagnosing TB as well. Two out of three culture negative false positive cases had culture negative clinical TB and responded well to treatment. This might be explained by absence of viable bacilli in these patients.[13] CBNAAT also helped in diagnosing a case of atypical mycobacteria which was smear positive but CBNAAT negative underlining its excellent specificity. The rate of invalid tests is lesser than culture contamination rate as was seen in our study

One sixth of the CBNAAT positive patients in our study were Rifampicin resistant and later confirmed to be MDR TB by LPA which is expected because 95% of cases of Rifampicin resistant turn out to be MDR TB.[16] However in view of smaller number of cases, these results should be confirmed in larger studies before extrapolating to populations with low prevalence of MDR-TB. Though maximum number of TB cases were detected in AIDS (CD4<200) group; the yield of smear positivity and CBNAAT positivity had a negative correlation with CD4 counts as has been documented before. This happens to decrease in cavitations and Acid fast bacilli load at lower CD4 counts. [27-30]

The strength of our study is that we included all patients presenting to HIV clinic without pre selection and sent the sample for processing without delay to a quality assured lab. We also compared it with commonly used symptom screen , chest x ray, sputum microscopy and diagnostic algorithm and generated test characteristics for every one of them. The limitation of our study is that we could use only one sputum for CBNAAT processing due to financial constraints and the number of Rifampicin resistant patients was too small to generalize its prevalence to a larger population. We used CBNAAT-TB for diagnosis of pulmonary TB only as recommended by WHO due to variable performance in detection of extra pulmonary TB . A recent meta-analysis of CBNAAT-TB, however showed modest sensitivity and specificity (80% and 86%) for detection of extra pulmonary TB. In HIV positive patients having TB, half of the patients with 20% of the patients have extrapulmonary TB only, 30 % have Pulmonary TB only while half of the patients have coexistent extrapulmonary and pulmonary TB. [30] Thus, Future studies should focus on performance of CBNAAT-TB in detection of extrapulmonary TB in HIV positive patients, as almost 20% HIV-TB co infection is purely extrapulmonary of this population.

Thus we conclude that CBNAAT-MTB has rightfully been claimed as game changer in the diagnosis of HIV-TB co infection. [18] It outperformed sputum microscopy, chest X-ray and all other clinical diagnostic algorithms in diagnosis of pulmonary TB in HIV patients in our study by a wide margin. Hence, it should be used as a first line test for diagnosis of TB in all HIV infection patients as advocated by WHO.

TABLE1. Demographic profile of Patients in the Study.

	Mean	95% CI	SD
AGE	27.877	26.143 - 29.612	12.5670
SEX(male)%	0.529	46.0 – 59.8	50.04
CD4(cell/cu mm)	215.5	205.14 - 225.85	75.0094
WEIGHT(kg)	41.637	39.954 - 43.321	12.1937
Chronic Cough(%)	0.377	31.0 - 44.5	48.59
Abnormal chest X-ray(%)	34.8	28.2 - 41.4	47.75
Positive smear (%)	15.7	10.7 - 20.7	36.46
Positive CBNAAT (%)	31.4	25.0 - 37.8	46.51
WHO symptom screen (%)	63.2	56.6 - 69.9	48.34
Abnormal chest X ray or symptoms (%)	68.1	61.7 - 74.6	46.71
Culture positive TB(%)	34.8	28.2 - 41.4	47.75
Any TB (culture positive and clinical TB)(%)	41.2	34.4 – 4.80	49.34
Patients on HAART(%)	55.3	43.4- 66.8	41

Duration of HAART (months) 4.25 3.77- 4.73 3.25
(CI = Confidence Interval, SD = Standard deviation)

Table 2. Comparative test characteristics of various symptoms and tests with culture positive Tuberculosis as gold standard.

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	PLR (%)	NLR (%)
CBNAAT-TB	85.92	97.74	95.3	92.9	38.2	0.14
Smear microscopy	39.44	96.99	75.0	87.50	13.11	0.62
Chest X ray	63.4	80.45	60.2	79.3	3.24	0.46
Chronic cough	53.5	70.7	49.35	74.02	1.83	0.66
WHO symptom screen	88.73	50.38	48.84	89.33	1.79	0.22
Symptom screen and chest X ray	91.55	44.36	46.76	90.77	1.65	0.19

(PPV=positive predictive value, NPV=negative predictive value, PLR=Positive likelihood ratio, NLR=Negative likelihood ratio)
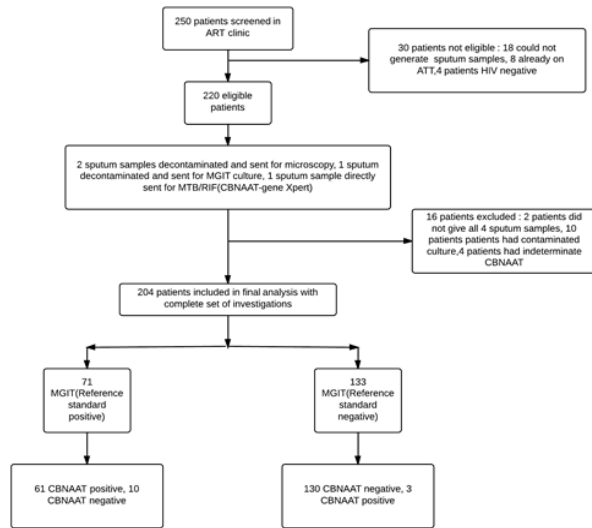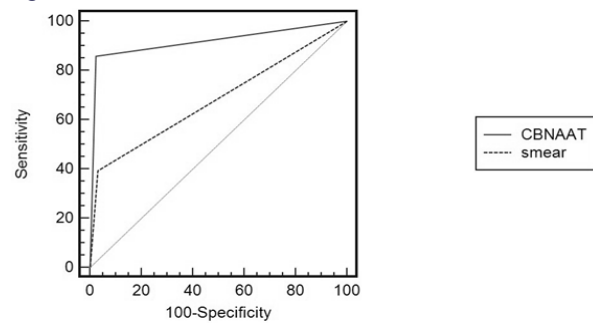

Figure 1.

Figure 2. Comparison of Receiver operator characteristic curves of Cartridge based Nucleic Acid Amplification test and smear microscopy for detection of Pulmonary Tuberculosis
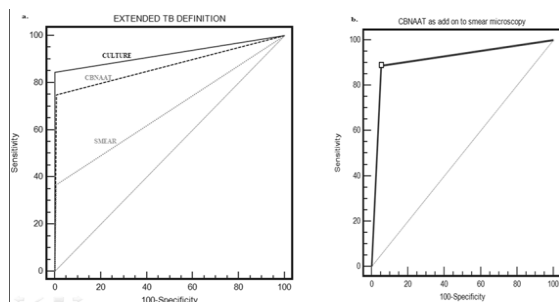

Figure 3.
a) Performance of various tests using an extended Tuberculosis definition on Receiver operator characteristic curve.
b) Performance of Cartridge based Nucleic Acid Amplification test as add on to sputum negative microscopy for diagnosis of Pulmonary Tuberculosis with Culture positive as Gold Standard

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