Original Research Article

A comparative study of the diagnostic efficacy of CB-NAAT and ZN staining

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Abstract

Background: In recent years,cartridge based nucleic acid amplification test(CBNAAT)has been recommended by World Health Organization as a rapid diagnostic test for detection of tuberculosis and rifampicin resistance. **Aim:** In this study, we retrospectively compared results for diagnosis of suspected pulmonary and Extrapulmonary tuberculosis cases, from March 2020 to September 2020 for Ziehl-Neelsen(ZN)and GeneXpert (Xpert®MTB/Rif assay)taking culture as gold standard. **Methods:** Performance of Xpert was compared to acid-fast microscopic examinationusingZiehl-Neelsen (ZN) stain in patients with culture-confirmed tuberculosis.**Results:** Out of total 914 specimens of clinically suspected patients of tuberculosis of all age groups,683(75%) were pulmonary specimens and231(25%)were extrapulmonary. For pulmonary samples, the sensitivity and specificityfor CBNAAT samples were 82.3%and98.5%while that for sputum smear were 63.7% and 99.3% respectively.For extrapulmonary samples,the sensitivity and specificity for CBNAAT samples were 85.7% and 93.5% respectively; while that for sputum smear were 60.7%and100% respectively. **Conclusion:** Although the development of the Xpert MTB/RIF assay is undoubtedly alandmarkeventina high TB burden country like India as this test will help in rapiddiagnosis of smear-negative and rifampicin resistant TB cases, which were earlier a challenge for the TB control programmes

Keywords: Tuberculosis (TB) Cartridge based nucleic acid amplification test (CBNAAT) Rifampicin (RIF), Mycobacterium tuberculosis(MTB),Ziehl Neelsen(ZN).

Introduction

According to WHO global TB report, the estimated incidence of TB (including TB with HIV) is 2.2 million and prevalence is 2.5 million with mortality (excluding TB with HIV) of 0.22 million[1-3]. India,one of the countries with high burden of TB, has an estimated 79,000 MDR- TB cases among notified pulmonary TB cases. The estimated incidence of -TB is 2% among new cases and 15% among re-treatment cases. Annually,one fourth of the global incident TB cases occur in India[4,5]. Early and accurate diagnosis is the first critical step in controlling TB. MDR The control of TB is hampered by diagnostic methods with sub- optimal sensitivity, particularly for the detection of drug resistant forms and in patients with human immunodeficiency virus (HIV) infection.

Early detection is essential to interrupt transmission and reduce the death rate, but the complexity and infrastructure needs sensitive methods which limit their accessibility and effect[2,3]. Diagnosis of pulmonary tuberculosis (PTB) mostly relies on the identification of acid-fast bacilli (AFB) in sputum smear, but its limitation is low sensitivity[2,3]. Conventional mycobacterial cultures (Solid culture in Lowenstein-Jensen medium) takes about 6-8 weeks' time; newer liquid culture methods like BACTEC or Mycobacterial growth indicator tube (MGIT) gives relatively rapid results but is costly[4,5].

Diagnosis of extrapulmonary tuberculosis (EPTB) remains especially challenging since the number of Mycobacterium tuberculosis (MTB) bacilli present in tissues at sites of disease is often low and clinical specimens from deep- seated organs may be difficult to obtain. Histology is time-consuming to undertake

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and establishing a diagnosis of TB with high specificity remains difficult. Tissue microscopy after special staining is often negative and when mycobacteria are seen, it is impossible to distinguish MTB from nontuberculous mycobacterial disease. Reliance on culture, the mainstay of diagnosis, often leads to considerable delays, compromising patient care andoutcomes[5-8]. To address this issue The Xpert MTB/RIF a Cartidge based nucleic acid amplification test (CBNAAT) assay was rapidly endorsed by the WHO in December 2010 , for diagnosis of tuberculosis with special emphasis on drug-resistant tuberculosis, human immuno-deficiency virus (HIV) and TB co-infection, paediatric tuberculosis, extrapulmonary tuberculosis and smear-negative pulmonary tuberculosis[9-11]. The Xpert® MTB/RIF assay (Cepheid Inc., CA, USA) marks an important development in the field of rapid molecular TB diagnostics This multifunctional diagnostic platform is an automated, closed system that performs real-time PCR and can be used by operators with minimal technical expertise, enabling diagnosis of TB and simultaneous assessment of rifampicin resistance to be completed within 2 h[11].

Since Xpert MTB/RIF was specifically developed and optimized for testing sputum samples and initial large-scale evaluations were in patients with pulmonary TB, WHO endorsement specifically applied to the investigation of pulmonary TB. More recently, however, evaluations of the assay have extended to a variety of nonrespiratory clinical samples from patients with EPTB [12]. However, compared with pulmonary disease, investigation for use in EPTB is far more complex because of the diversity of clinical sample types, difficulties in obtaining adequate tissue for analyses and in extraction of MTB DNA from samples, the challenge of providing a rigorous gold standard for comparison, and therange of potential ways of processing samples prior to analysis [12,13].Therefore the aim of this study was to determine the diagnostic efficacy of CBNAAT in pulmonary tuberculosis (PTB) and extrapulmonary cases and to compare its efficacy between smear-positive and smear-negative pulmonary and extrapulmonary tuberculosis.

Material and methods

Study design

This was a single-center, prospective, and comparative study. The culture positive samples from patients with clinical, radiological suspicion of pulmonary and extrapulmonary tuberculosis studied in the Department of Microbiology, at Patna Medical College and Hospital, Patna from March2020 to September 2020, with smear microscopy Ziehl-Neelsen (ZN) and CBNAAT.The study was approved by the Institutional Research Committee. An informed and written consent was obtained from all the participating subjects before the commencement of the study.

Sample Processing

Non sterile specimens were processed by modified petroff method. After decontamination, sediment was dissolved in 2.5ml of distilled water for microscopy and inoculation in culture medium. Sterile specimens were concentrated by centrifugation and smear and cultures was inoculated from the sediment[13].

Tissue

The tissue was homogenized in a tissue grinder with a small quantity of sterile saline or water (2-4ml).the homogenized specimen was decontaminated using NALC-NaOH procedure as in sputum.

Resuspend the sediment with phosphate buffer. If the tissue grinder was not available, a mortar and pestle was used. Tissue was also placed in a Petri dish with sterile water (2-4 ml) and torn apart with the help of two sterile needles.Smears were prepared and stained with. ZeihlNeelson staining method and was graded as per RNTCP guidelines: Scanty (1-9/100 fields), 1+ (10-99/100 fields), 2+ (1-10/ fields) and 3+ (>10/field). A person was taken as smear positive if at least one of the smears was graded scanty or higher.7 Culture was done on either solid media (LJ media)) using standard protocol[13-15].Specimen was inoculated on the LJ medium and incubated at 37ºC for growth. Cultures were incubated for 8 weeks in case of solid culture. Contamination by rapidly growing bacteria and those with morphologies inconsistent with MTBC were checked regularly. After the appearance of growth on LJ medium, identification of M. tuberculosis was done by morphological examination, ZN staining and biochemical tests[11-14].

Analysis of samples by Xpert MTB/RIF assay

The assay was performed using version 4 cartridges according to the manufacturers' recommendations. Briefly the sample reagent (containing NaOH and isopropyl alcohol) was added at a 2:1 ratio to clinical specimen to kill the mycobacteria and liquefy the samples. For biopsy specimen, a 2:1 volume of sample reagent (SR) buffer was added to biopsy specimens after they had been chopped into very small pieces with a sterile blade in a sterile petridish Fluids were processed directly by the addition of a 2:1 volume of SR buffer, except for CSF (usually <1ml), which was

raised to 2ml by the addition of SR buffer. The sample-SR mixture was shaken vigorously and incubated for 10 minutes before being shaken again and kept at room temperature for another 10 minutes. Two ml of the digested material was transferred to the cartridge. The cartridge was subsequently loaded in the GeneXpert instrument where allsubsequent steps occurred automatically. In case the results were reported as invalid, error or no result, the sample was reprocessed and rerun, if sufficient material was available[15-17].

Data Collection

The data collected included the patients' demographics, semi quantitative bacillary load by AFB microscopy and past history of TB treatment

Statistical Analysis

The patients were characterized using simple descriptive statistics. Sensitivity, specificity, positive predictive value, and negative predictive value of smear microscopy and the Xpertassayfor detecting MTBC was done using phenotypic culture as the reference standard.

All the specimens which were culture positive and mycobacterium tuberculosis/resistance to rifampicin (MTB/RIF) assay negative and specimens that were culture negative and TB/RIFassay positive were retested twice. The last result was included for the analysis.Diagnostic accuracy was first assessed by simple comparison with mycobacterial culture results. Analysis was then made against a TB diagnostic gold standard that incorporated all culture- positive diagnoses.

Result

Of the total 914 clinically suspected tubercuosis specimens ,were collected, in which 683(75%) were pulmonary which included sputum (594/ 87%), bronchoalveolar lavage (27/4%), and gastric fluid specimens (62 /10%)(Figure1, Table1) and 231(25%) were presumptive extra pulmonary tuberculosis received from different anatomical sites were: tissue biopsies or fine- needle aspirates (65 /28%), pus (56/24.2%), pleural fluid (55/24%, other body fluids (peritoneal, synovial and pericardial: 24/10.4%) CSF (23/11.3%), endometrial biopsy (4/1.7%).) (Figure2, Table1) . 539 were male, with Male to Female in ratio(1:1.4)(figure3). Maximum clustering was seen in 20-50) agegroup (Figure 4).

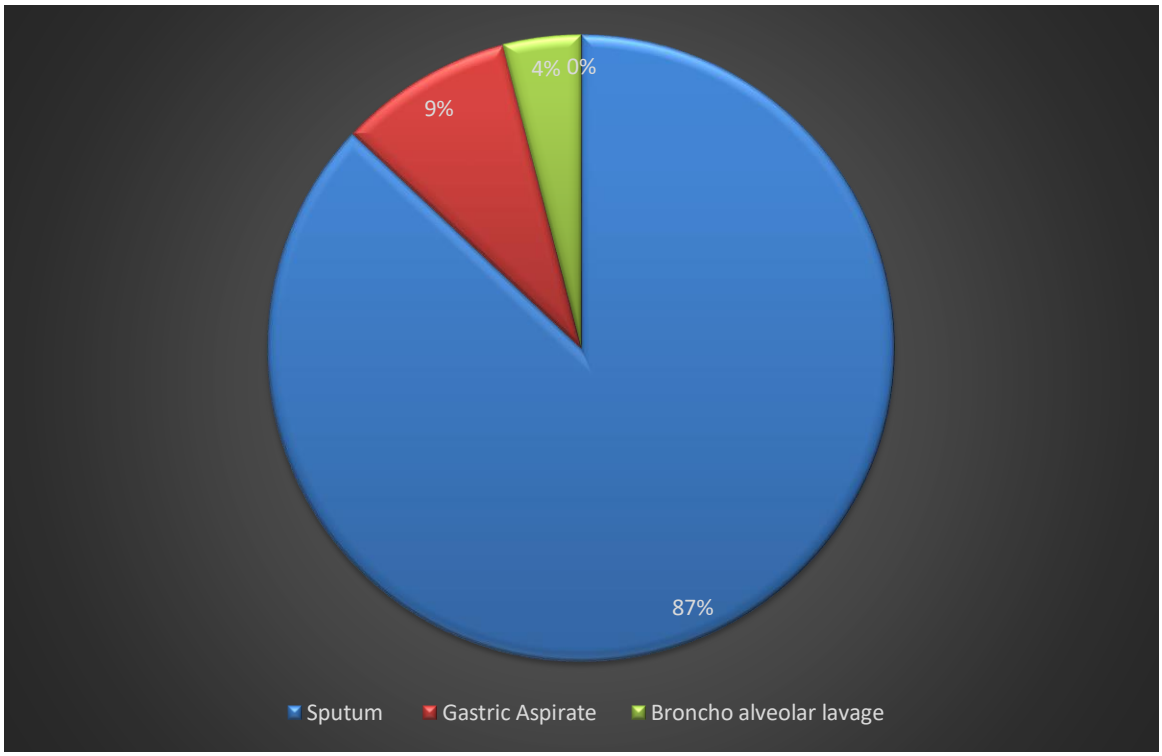

Fig 1:Distribution of pulmonary samples

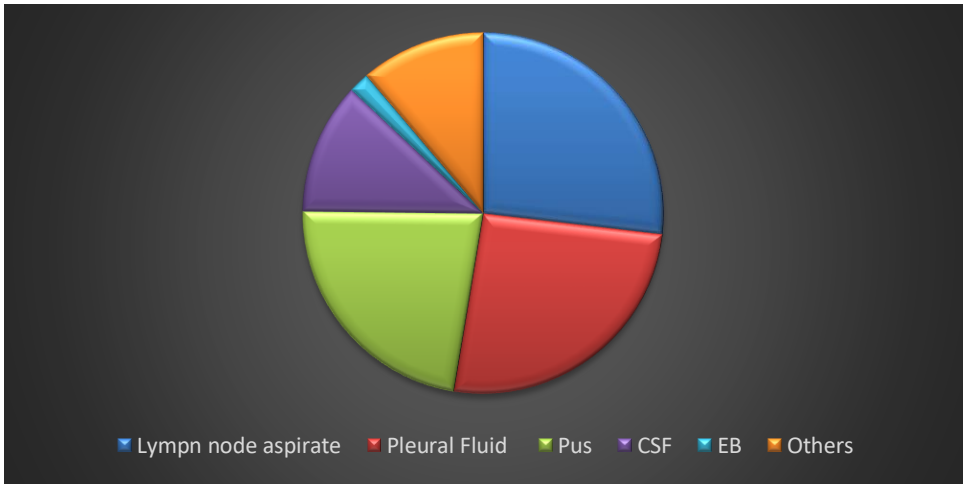

Fig 2:Distribution of extra pulmonary samples

Table 1: Distribution of pulmonary and Extrapulmonary samples

Pulmonary samples (n =683)	Number of samples %
Sputum	594/87%
Gastric aspirate	62/10%
Bronchoalveolar lavage	27/3%
Extraplmonary Samples (n =231)	
Tissue aspiration/Biopsies	65/28%
Pus	56/24%
CSF	23/11%
Pleural fluid	55/24%
Other aspiratesKnee aspirates,ascitic aspirates etc.	24/10%
Endometrial biopsy	4/2%

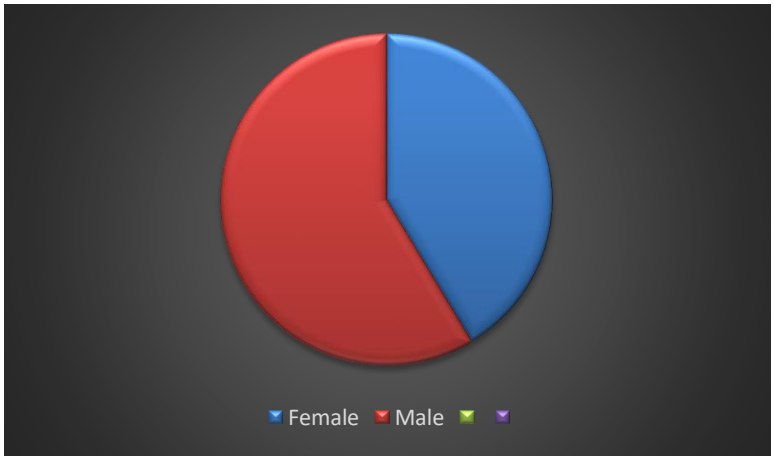

Fig 3:Sex wise distribution

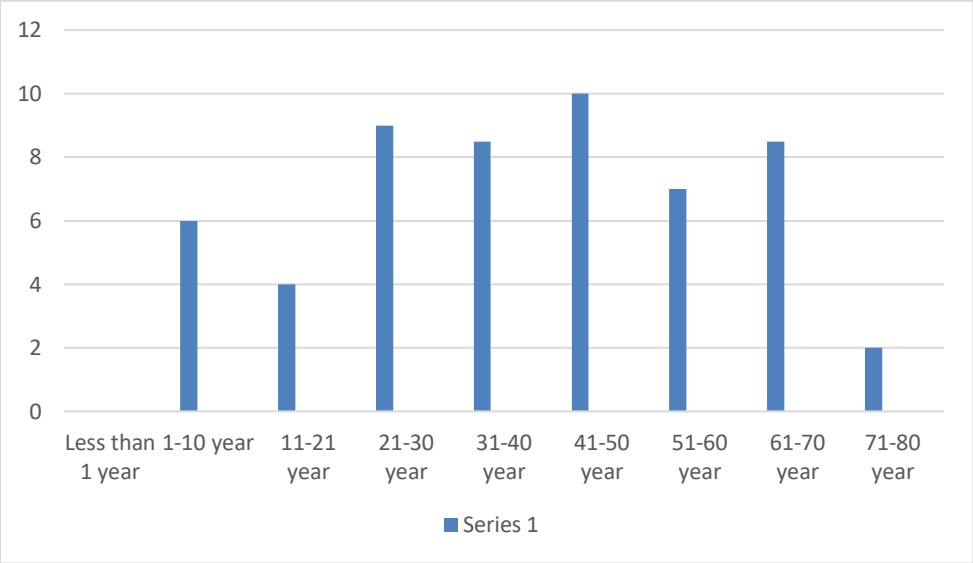

Fig 4: Age wise distribution

In the present study, out of the 914 samples 250 were Mycobacterium tuberculosis culture confirmed samples.176 were pulmonary and 74 were extrapulmonary. In culture confirmed pulmonary samples 146 were detected by CBNAAT and 118 by microscopy. 35 were CBNAAT positive and smear negative (Table 1) Sensitivity and Specificity for CBNAAT(Catridge based nucleic acid based amplification) :82.3% and 98.5% Sensitivity and Specificity for sputum smear microscopy(Ziehl Neelsen) 63.7% and 99.3%% respectively.(Table 2). Maximum positivity was seen in sputum samples by CBNAAT.(77.8%). Out of the 74culture confirmed extrapulmonary samples 61 (82.4%) were positive by CBNAAT and 28(37.8%) by microscopy and 36 were CBNAAT positive and microscopy negative. For extra-pulmonary maximum positivity was seen for Lymph Node aspiration(96.4%). 10/146 (5.6%) samples were found to be rifampicin resistance in pulmonary group and 5/61 (6.8%) in extrapulmonary group.

Table 3: Comparison of culture positive pulmonary and extrapulmonary samples in microscopy and CBNAAT

	Culture positive specimens	
	Pulmonary Specimens (n=176)	Extrapulmonary specimens (n=74)
Microscopy Positive	118	28
Microscopy Negative	58	46
CBNAAT Positive	146	61
CBNAAT Negative	30	13

Table 4: Sensitivity and Specificity in CBNAAT and Microscopy

	Pulmonary samples		Extrapulmonary samples	
	MICROSCOPY	CBNAAT	MICROSCOPY	CBNAAT
Sensitivity	63.7%	82.3%	37.84%	82.4%
Specificity	99.3%	98.5%	98.2%	100%

Discussion

India accounts for around one-fourth of the global tuberculosis cases[1]. Detection of AFB in sputum smear is a simple, rapid, inexpensive and very specific for diagnosis for PTB, its limitation is its low sensitivity[2,3]. Sputum culture for Mycobacterium tuberculosis is more sensitive and specific, but it takes 2-8 weeks' time depending on the method used and is costly[4,5]. Chest x-ray is neither sensitive nor specific for diagnosis of PTB[5]. So, there was a long felt need for a newer rapid diagnostic test for PTB with

improved sensitivity and specificity. WHO has endorsed the use of CBNAAT as a rapid diagnostic test for diagnosis of tuberculosis and prioritised areas like drug-resistant tuberculosis, paediatric tuberculosis, TB-HIV co-infection, extrapulmonary tuberculosis and sputum smear- negative PTB for use of CBNAAT [9,11].Our study findings suggest that CBNAAT hashigher sensitivity for detection of pulmonary and extrapulmonary tuberculosis cases. The WHO 2012 has also recommended the CBNAAT for routine use under programmatic conditions[9].The sensitivity of CBNAAT in smear-positive, culture-positive and smear-negative, culture- positive pulmonary samples were 100% and 66.67% respectively. Sensitivity of smear negative pulmonary samples can be increased by including more than one sample for diagnosis.In a study done by Panayotis et al[18] the sensitivity and specificity of CBNAAT in 80 pulmonary samples were 90.6% and 94.3% respectively. In a study done by Armand et al[19] the sensitivity of CBNAAT in 60 pulmonary samples which included sputum, BAL, bronchial aspirate and gastric aspirate was 79%. Among individual extrapulmonary samples, the sensitivity of CBNAAT was highest among lymph nodes (96.74%) when compared to sputum smear (77.8%). Inclusion of CBNAAT in the initial diagnosis of tubercular lymphadenopathy in addition to the FNAC would decrease the over diagnosis of tuberculosisand injudicious use of anti-tuberculosis treatment (ATT).Various studies conducted across India has suggested the usage of CBNAAT up-front for people living with HIV (PLHIV) [15]. The operational feasibility studies conducted under the Revised National TB Control Programme (RNTCP) have demonstrated the feasibility of the machine to efficiently work under Indian settings[6].In a study done by Subhakar Kandi et al[20] Department of Pulmonary Diseases Osmania Medical College, the sensitivity of CBNAAT for pulmonary samples was 79% when compared to sputum smear which was 42%. The sensitivity of CBNAAT for extrapulmonary samples was 86% when compared to sputum smear which was 61%[17]. In another study by Zahoor D et al[22]

122 pulmonary samples and 153 extrapulmonary samples collected from 275 patients were included in the study. Out of these, 48 samples were positive by both culture and Xpert assay and 2 samples were culture positive only. Out of 225 culture negative samples, 3 were positive by GeneXpert. The sensitivity for GeneXpert was much higher compared to smear micrscopy(96 Vs 46% respectively). The Xpert assay also detected 3 rifampcin resistantcases.Our study findings suggest that CBNAAT hashigher sensitivity for detection of pulmonary and extrapulmonary tuberculosis cases. The WHO 2012 has also recommended the CBNAAT for routine use under programmatic conditions[1].In the present study, only 914 specimens were included; among them 683 were pulmonary and231 were extra-pulmonary. Among the 683 pulmonary presumptive TB cases,. The sensitivity of CBNAAT for pulmonary samples was 82.3% when compared to sputum smear which was 63.7%. sensitivity of CBNAAT for extrapulmonary samples was 82.4% when compared to sputum smear which was 37.84%. Out of the 58 smear negative pulmonary samples, CBNAAT has detected 35 because of pausi bacillary nature of the sample. Out of the 146 CBNAAT positives rif sensitive was 136 and 10(5.6%) were rif resistance. In extrapulmonary samples out of 46 smear negative samples CBNAAT detected 27 samples and rif resistance was detected in 5 (6.8%)samples.This suggests that CBNAAT is a sensitive tool to detect TB in smear negative pulmonary and extra pulmonary tuberculosis, with rifampicin resistance.

Conclusion

CBNAAT / Xpert MTB/RIF assay is undoubtedly a landmark event and one of the rapid diagnostic tests available in the country.It can serve as a sensitive and time-saving diagnostic method for micro-biological diagnosis of TB as it requires minimal expertise and handling especially in remote rural settings. It should be routinely used under the public and private health sectors efficiently to detect a tuberculosis case but still its clinical and programmatic effects and cost-effectiveness still remain to be defined. This multifunctional diagnostic platform is an automated, closed system that performs real-time PCR and can be used by operators with minimal technical expertise, enabling diagnosis of TB and simultaneous assessment of rifampicin resistance to be completed within 2 h .It is one of the rapid diagnostic tests available in the country and it should be routinely used under the public and private health sectors efficiently to detect a tuberculosis case.

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