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Comparative study of nested PCR and CBNAAT in patients of pulmonary tuberculosis

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Abstract
Aim: comparative analysis of NESTED PCR and CBNAAT in patients of pulmonary tuberculosis.
Materials and methods: this comparative study was conducted at Sri Guru Ram Das Institute of Medical Sciences and Research, Sri Amritsar on 60 pulmonary tuberculosis patients who include 30 patients who were sputum positive and 30 sputum negative patients. Routine blood examination including and sputum smear examination was done in these patients. Further evaluation was done by CBNAAT and nested PCR
Results: Overall mean age of study population was 45.52±19.15 years (mean ± SD). 61.7% (37 out of 60) patients were male. Among sputum positive group overall sensitivity of CBNAAT and Nested PCR was 88.0% (68.78, 97.45), 74% (47.62, 92.73) with specificity of 80.0% (28.36, 99.49), 21.43% (4.66, 50.80) and sputum negative group overall sensitivity of CBNAAT and Nested PCR was 85.71% (63.66, 96.95), 46.67% (21.27, 73.41) with specificity of 77.78% (39.99, 97.19), 13.33% (1.66, 40.46).
Conclusion: study concluded that Xpert/CBNAAT has high sensitivity and specificity for diagnosis of both smear positive and smear negative cases when compared with NESTED PCR.

Keywords: NESTED PCR, Xpert/CBNAAT, pulmonary tuberculosis

Introduction
Tuberculosis is a great menace to mankind since ancient times. The genus Mycobacterium was known to originate 150 million years ago. The skeletal deformities of tuberculosis have been demonstrated in Egyptian mummies [1]. But the causative agent of this dreadful disease was not known for longer periods. The discovery of Mycobacterium tuberculosis, the causative agent for tuberculosis was by Robert Koch in 1882 using light microscopy and a special staining technique [2].
In 2017, TB caused an estimated 1.3 million deaths (range, 1.2–1.4 million) among HIV-negative people and there were an additional 300 000 deaths from TB (range, 266 000–335 000) among HIV-positive people. Globally, the best estimate is that 10.0 million people (range, 9.0–11.1 million) developed TB disease in 2017: 5.8 million men, 3.2 million women and 1.0 million children. There were cases in all countries and age groups, but overall 90% were adults (aged ≥15 years), 9% were people living with HIV (72% in Africa) and two thirds were in eight countries: India (27%), China (9%), Indonesia (8%), the Philippines (6%), Pakistan (5%), Nigeria (4%), Bangladesh (4%) and South Africa (3%) [3].
Till now diagnostic tests for *tuberculosis* include sputum smear analysis (the microscopic examination of mucus brought up from the lungs by coughing for the presence of *Mycobacterium tuberculosis*) and mycobacterial liquid culture.
For several decades smear microscopy and conventional culture techniques have been the mainstay of diagnostic testing for pulmonary tuberculosis. While smear microscopy has poor sensitivity and issues related to quality control. Conventional solid culture techniques have the limitation of long turnaround time of several weeks. Liquid culture techniques were developed for early detection of Mycobacterium tuberculosis growth, but the mean turnaround time of 21 days is still long for a diagnostic test to be effective in curbing transmission. Such delays in diagnosis increase morbidity and mortality predispose to secondary resistance and cause transmission of resistant strains [4].
During the last decade, a number of nucleic acid amplification (NAA) methods have been developed for rapid detection and identification of Mycobacterium tuberculosis (MTB) in clinical specimens. These techniques are attractive because they allow for the direct detection of low Mycobacterium tuberculosis (MTB) genomic copy numbers in specimens. Polymerase chain reaction (PCR) is based on NAA methods and is widely used for the rapid diagnosis of tuberculosis [5]. Due to paucity of the data in the literature the present study is carried to compare nested PCR and CBNAAT in patients of pulmonary tuberculosis of Punjab, India.

Materials and Methods
This study was conducted at Sri Guru Ram Das Institute of Medical Sciences and Research, Sri Amritsar on 60 pulmonary tuberculosis patients who include 30 patients who were sputum positive and 30 sputum negative patients, in collaboration with the Department of Microbiology.

Inclusion Criteria
- Patients with clinical suspicion of Pulmonary tuberculosis based on symptoms (e.g., cough more than

two weeks, hemoptysis, fever, asthenia, loss of weight and night sweats)
- Radiological features (e.g., nodule, consolidation, cavitation and other opacities)

Exclusion criteria
- Patients who were on Anti tubercular treatment for more than 2 weeks were excluded from the study.

Investigations
Routine blood examination including CBC, FBS, HIV, LFT, RFT, Urine R/M, Chest X-Ray and sputum smear examination was done in these patients.

Technique
Sputum was collected from these patients and further evaluated by CBNAAT and nested PCR.

Nested PCR
This test is based on the principles of single-tube nested PCR method, which is a powerful and sensitive diagnostic tool for the identification of Mycobacterium Tuberculosis complex. This assay is a two- step sequential assay. In the first step, the IS region of Mycobacterium Tuberculosis complex DNA sequence, a 220 bp is amplified by specific external primers. In the second step, the nested primers were added to further amplify a 123 bp amplification product. In this test, false positive reactions that may be caused by previous amplication contamination are prevented by the use of Uracil DNA glycosylate (UDG) and Di-uracil dnaglycosylate (DTP) instead of Deoxyuridine triphosphate (DTTP) [6].

CBNAAT
The assay utilizes single-use plastic cartridges with multiple chambers that are preloaded with liquid buffers and lyophilized reagent beads necessary for sample processing, DNA extraction and heminested RT-PCR.
Clinical sputum samples (or decontaminated sputum pellets) were treated with sodium hydroxide and isopropanol-containing sample reagent (SR). The SR will be added to the sample (currently recommended at a 3:1 ratio for sputum pellets and a 2:1 ratio for unprocessed sputum samples) and incubated at room temperature for 15 min. The treated sample was manually transferred to the cartridge that will be loaded into the CBNAAT instrument.
Subsequent processing is fully automated. The cartridge incorporates a syringe drive, a rotary drive and a filter upon which Mycotuberculosis bacilli are deposited after being liberated from the clinical material. The test platform employs a sonic horn that inserts into the cartridge base to cause ultrasonic lysis of the bacilli and release of the genetic material. The assay then amplifies a 192 bp segment of the rpoB gene using a hemi-nested RT-PCR reaction [7].

Statistical analysis
The data was analyzed using SPSS 19 (SPSS Inc. Chicago, IL, USA) Windows software program. Descriptive frequencies were expressed using mean and standard deviation. Sensitivity and specificity were calculated with 95% confidence interval (CI) where relevant.

Results
Table 1: depicts the demographic details of the study population. Overall mean age of study population was 45.52±19.15 years (mean ± SD). 61.7% (37 out of 60) patients were male. Majority (76%) of the patients in the study were farmers (31.7%) by occupation.
Table 2: depicts the prevalence of symptoms in the study population. Out 0f 60 patients Fever (n=44) and cough (n=42) were the most prevalent followed by breathlessness (n=20) and hemoptysis (n=14).
Table 3: among sputum positive group overall sensitivity of CBNAAT and Nested PCR was 88.0% (68.78, 97.45), 74% (47.62, 92.73) with specificity of 80.0% (28.36, 99.49), 21.43% (4.66, 50.80)
Table 4: among sputum negative group overall sensitivity of CBNAAT and Nested PCR was 85.71% (63.66, 96.95), 46.67% (21.27, 73.41) with specificity of 77.78% (39.99, 97.19), 13.33% (1.66, 40.46).

Table 1: Demographics of the study population

Age	Frequency (n)	Percentage (%)
15-24 Yr	12	20.0
25-34 Yr	9	15.0
35-44 Yr	7	12.0
45-54 Yr	9	15.0
55-64 Yr	10	17.0
65-74 Yr	8	13.0
>74 Yr	5	8
Mean±SD	45.52±19.15	
Gender		
Female	23	38.3
Male	37	61.7
Occupation		
Driver	4	6.7
Farmer	19	31.7
Housewife	12	20.0
Shopkeeper	6	10.0
Student	9	15.0
Worker	10	16.6
Total	60	100.0

Table 2: Distribution of symptoms

Symptoms	Frequency (n)
Cough	42
Hemoptysis	14
Breathlessness	20
Fever	44

Table 3: Diagnostic Accuracy of Nested PCR and Xpert/CBNAAT for 30 sputum Positive cases (Culture Positive 23 & Negative 7)

Variables	Nested PCR	CBNAAT
Sensitivity, % (95% CI)	74 (47.62, 92.73)	88.0 (68.78, 97.45)
Specificity, % (95% CI)	21.43 (4.66, 50.80)	80.0 (28.36, 99.49)

Table 4: Diagnostic Accuracy of Nested PCR and Xpert/CBNAAT for 30 sputum Negative cases (Culture Positive 20 & Negative 10)

Variables	Nested PCR	Xpert/CBNAAT
Sensitivity, % (95% CI)	46.67 (21.27, 73.41)	85.71 (63.66, 96.95)
Specificity, % (95% CI)	13.33 (1.66, 40.46)	77.78 (39.99, 97.19)

Discussion

India accounts for around one-fourth of the global tuberculosis cases [8]. Detection of AFB in sputum smear is a simple, rapid, inexpensive and very specific for diagnosis for PTB, its limitation is its low sensitivity [9]. 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 [10]. Chest x-ray is neither sensitive nor specific for diagnosis of PTB [11]. 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 [12].

As an initial diagnostic method of M.TB, the accuracy and speed of the Xpert MTB/RIF assay have been demonstrated in previous studies. However, many of these were performed in high-income countries with sufficient medical resources. Our study was performed in India where medical resources such as solid and liquid mycobacterial culture systems are not readily available.

In our study, of 60 TB suspects, 43 patients were positive by mycobacterial culture. However, mycobacterial culture failed to identify M.TB in 17 patients who were concluded to have pulmonary TB based on the symptoms and radiographic findings. The fact that approximately one third of pulmonary TB cases could not be identified by mycobacterial culture suggests that more sensitive diagnostic methods were required.

In this study, mean age of PTB patients was 45.52±19.15 years (mean±SD) with male preponderance (61.7%). Dewan *et al*. [13] have also reported that mean age of patients in their study was 45.1 ± 9.4 years, 69% of their patients were in 20-40 years age group and 76% were male.

In our study, the Xpert MTB/RIF assay for diagnosis of pulmonary TB in both sputum positive and negative cases was 50 out of 60 patients [83%]. Our results were found in agreement with a recent meta-analysis reported sensitivity of 90.4% [14].

In a study conducted by Kwak N *et al*. [15] revealed that TAT of the Xpert MTB/RIF assay was shorter than AFB smears, mycobacterial culture and DST in terms of time to report of results. Consequently, the Xpert MTB/RIF assay has shortened the time to initiation of anti-TB drugs by median 14 days.

While Xpert MTB/RIF may be the foremost choice amongst all molecular diagnostic tests, it has its own limitations. Resistance to RIF is taken as a surrogate marker for MDR-TB, but certain strains may exhibit only mono-resistance to RIF that may not warrant full line MDR therapy, thus, leading to over-estimation of the MDR-TB cases. Likewise, a study from Mumbai, India demonstrated how specimens with rifampicin results reported as sensitive by GeneXpert could be resistant to isoniazid [16]. Other drawbacks of Xpert MTB/RIF are requirement of stable electrical power supply, temperature control and annual calibration of instrument. Regardless of all these limitations, addition of Xpert MTB/RIF assay to the present set of diagnostic modalities for TB on account of its unambiguous, rapid results, and high sensitivity and specificity will facilitate early diagnosis.

Conclusion

Our study concluded that Xpert/CBNAAT has high sensitivity and specificity for diagnosis of both smear positive and smear negative cases when compared with NESTED PCR. In resource-limited settings and less accessible areas where establishing a sophisticated laboratory for culture to the prescribed biosafety levels is difficult, Xpert/CBNAAT provides a viable option. Widespread application of this assay thereby facilitates early treatment decisions and curbing transmission. Thus, the present study opens new vista for more detailed research.

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