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ORIGINAL ARTICLE

Comparative Study of Traditional and Molecular Methods of Diagnosis and Resistance Determination in Paediatric Pulmonary Tuberculosis Samples

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Abstract

Background: The burden of childhood tuberculosis provides the measure of tuberculosis control in a community as it reflects recent transmission. The paucibacillary nature of the disease and the inability to produce sufficient volumes of sputum severely hamper accurate diagnosis of tuberculosis in children. It is also equally important to address drug-resistant tuberculosis. Cartridge-Based Nucleic Acid Amplification Test (CBNAAT) is one such test that aids in the rapid detection of Mycobacterium tuberculosis and also rifampicin resistance simultaneously. Very limited studies are available in pediatric cases of pulmonary tuberculosis on the efficacy of CBNAAT compared to other conventional and molecular methods. **Material & Methods:** 50 children less than 15 years of age suspected to have pulmonary tuberculosis according to National TB Guidelines 2012 were included in the study. Pulmonary samples such as sputum samples (n = 30) and gastric aspirates (n = 20) were collected and sent for testing. **Results**: Among the 50 subjects, 78% were Tuberculin skin test (TST) positive, 14% were positive for AFB microscopy, CBNAAT was positive in 80%, liquid culture was positive in 40%, and PCR was positive in 52%. Using CBNAAT, n = 34 patients were rifampicin sensitive, and n = 6 were rifampicin resistant. Conventional DST (n = 20) showed RR pattern (resistance to both INH and Rifampicin) in n = 3 and SS pattern (sensitivity to both INH and Rifampicin) in n = 17. LPA (n = 22) showed RR pattern in n = 3, SR pattern (INH sensitive/Rifampicin resistance) in n = 1, and SS pattern in n = 18. **Conclusion**: CBNAAT results are quite promising for the diagnosis of pulmonary TB in paediatric patients residing in highly TB-endemic countries compared to other tests. It is the least time-consuming test and has the extra benefit of simultaneous Rifampicin resistance detection, which will be a great aid for treatment planning.

Keywords: Children, Tuberculosis, CBNAAT, rifampicin resistance

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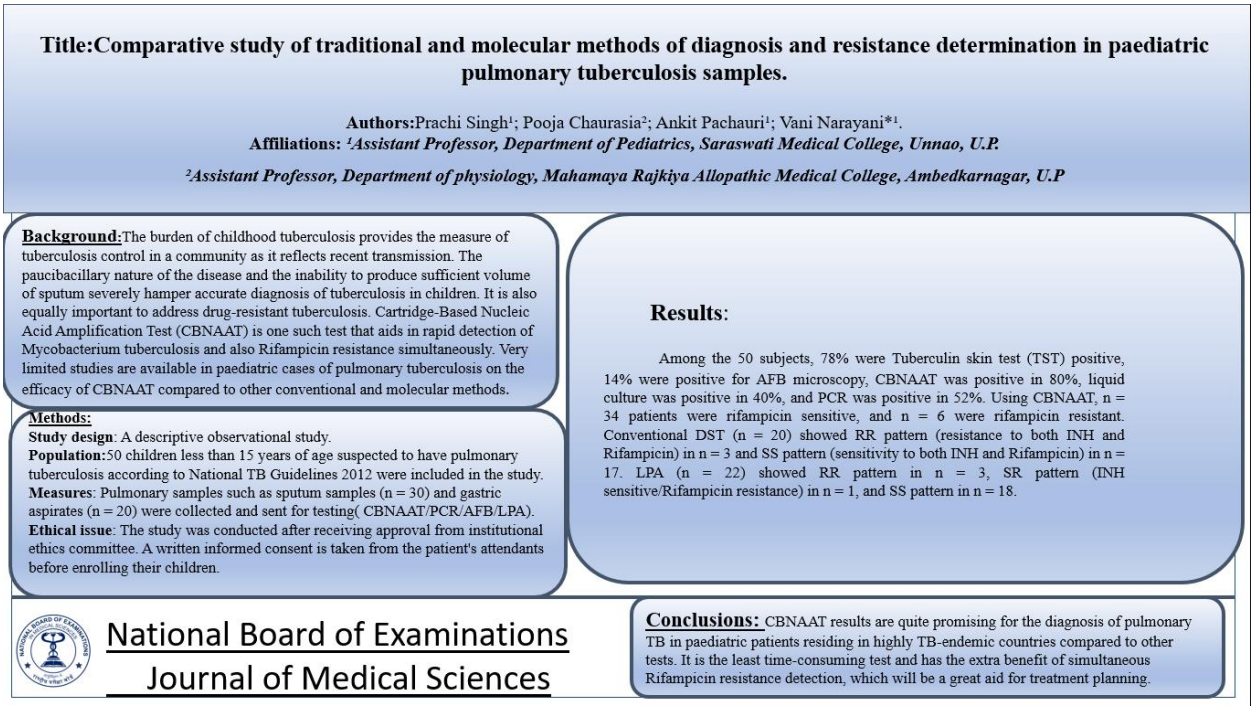
Graphical Abstract



Introduction

Mycobacterium tuberculosis is the pathogenic organism that causes tuberculosis. Roughly 6–8% of tuberculosis patients are under the age of fifteen. The burden of childhood tuberculosis indicates the measure of tuberculosis control in a community as it reflects recent transmission.

After HIV/AIDS, tuberculosis (TB) is the leading cause of death from a single infectious agent and the ninth most common cause of death globally. 10.4 million new cases of tuberculosis (TB) were reported worldwide in 2016, of which 10% were individuals living with HIV. An estimated one million children also became ill with TB. Ninety percent were adults, of whom 65% were male. Seven nations—India, Indonesia, China, the Philippines, Pakistan, Nigeria, and South Africa—accounted for sixty-four percent of the total [1]. Globally, there were 6 lakh cases of MDR-TB in 2016, including 1.1 lakh R-R cases. 47% of all MDR/RR-TB cases overall originated in China, India, and the Russian Federation. India and China accounted for 39% of the global gap. For not notified the DR-TB, it is estimated XDRTB is of 6.2% of MDR cases. It is now believed that TB is a major or contributory cause of many deaths in children under five years old [2]. As per the Global TB Report 2017, the estimated incidence of TB in India was around 2,800,000 accounting for about a quarter of the world's TB cases. Out of them, 423,000 die due to TB [3]. The burden of childhood TB in India was little known; however, according to WHO regional data, 0.6%–3.6% of all cases reported were sputum microscopy smear-positive in children under the age of 14. However, these results underestimate the true burden of childhood tuberculosis (TB) because the majority of

children were sputum microscopy smear negative. According to estimates, 8–20% of TB-related deaths occur in high-burden countries, where childhood TB accounts for 10–20% of all cases of TB [4].

According to the WHO, detection of TB disease in children is often unnoticed because it has non-specific symptoms and is difficult to diagnose. In India, sputum smear microscopy remained the mainstay for diagnosing pulmonary TB. However, because the disease is paucibacillary, there are insufficient bacteria in sputum and stomach aspirate samples for microscopy to detect the bacilli [5].

253 pulmonary and 176 extrapulmonary specimens were collected and tested in the MTB/RIF assay in a study conducted by Zeka et al. Sensitivities for smear-positive and smear-negative lung specimens were 100% (27/27) and 68.6% (24/35) respectively. In extrapulmonary specimens, its sensitivity was reduced to 100% in smear-positive specimens (4/4) and 47.7% in smear-negative specimens (21/44). The GeneXpert test has a very high specificity (97–100%) when it comes to diagnosing pulmonary tuberculosis, according to Nicol et al.

Although pulmonary TB is the most prevalent in children, extra pulmonary TB accounts for 20–30% of all paediatric cases. Among paediatric patients with pulmonary tuberculosis, the prevalence of drug resistance is also on the rise (6). This shows how important diagnosis of TB in children is, the purpose of this study was to compare CBNAAT against all other conventional and molecular methods that our centre offers.

Aims and objectives

The study aimed to detect *Mycobacterium tuberculosis* in pulmonary clinical specimens (sputum/gastric aspirates) using Gene Xpert testing (CBNAAT), PCR targeting IS6110, liquid culture MGIT 960, and conventional methods like CXR, TST, and AFB smears. To detect drug resistance to *Mycobacterium tuberculosis* using Gene Xpert CBNAAT, conventional DST, and LPA and compare the results. We have made an attempt to compare the CBNAAT results with both conventional and molecular methods of diagnosis and resistance detection in paediatric pulmonary TB samples. Also, the results of all studies with respect to age and sample type (sputum/gastric aspirate) have been compared.

Material and Methods

The study included children (0–15 years) of either sex who were suspected to have Pulmonary tuberculosis, according to the National TB Guidelines 2012, was selected for the study. Those patients who were already on anti-tubercular drugs were excluded from the study. A written informed consent is taken from the patient's attendants before enrolling their children. All the cases were subject to detailed clinical history, contact history with tuberculosis patients, immunisation history, socioeconomic classification according to the modified Kupuswamy scale, and history of previous chemotherapy. A thorough clinical examination was done, and anthropometry measurements were recorded to assess the nutritional status. TST testing and chest X-rays were done in all cases. Gastric aspirate/sputum collected from the study

subject was subjected to AFB microscopy using Ziehl Neelson staining, CBNAAT (cartridge-based nucleic acid amplification test also known as GeneXpert test), PCR IS6110 (polymerase chain reaction targeting insertion sequence 6110), line probe assay, culture on the BACTEC MGIT 960 TB system, and conventional DST. A 10 ml sample (sputum/gastric aspirate) was collected from each subject. 5 ml was sent to the state TB training and demonstration centre, and a 5 ml sample was sent to the National JALMA Institute. TRANSPORT-Specified samples were collected in sterile vials under aseptic conditions and stored at 2-8°C. The samples were then transported to the laboratory at <8° maintaining the cold chain.

Analysis: All the data were collected and compiled in an MS Excel spreadsheet. Data were analysed using SPSS software version 20.0. Descriptive data was expressed in the form of frequency and percentages. Sensitivity (Sn), specificity (Sp), positive predictive value (PPV), and negative predictive value (NPV) of different tests were also calculated. Applying the chi-square test, P values were obtained.

Results

In the current study, 50 children suspected to have pulmonary TB were included. 56% of the patients were males (n = 28), and 44% (n = 22) were females. The majority (40%) of the patients were 15 years old, followed by 11–15 years (26%), 6–10 years (24%), and less than one year (10%). Among male patients (n = 28), the majority of the patients were 1–5 years old (42.9%), while among female patients (n = 22), the majority of the patients were 11–15 years old (40.9%). More than half (52%) of the patients in the current study belonged to the middle class. 40 patients (80%) had history of contact with TB patients.

In our study, 98% of cases were malnourished. According to the WHO classification, 42% of cases were in moderate malnutrition and 18% were severely malnourished. Chest roentgenography was abnormal in the majority of cases in our study, showing hilar lymphadenopathy in 46% of cases. Other findings were patchy opacities in the lung parenchyma (34%),, pleural effusion (2%), miliary shadows (2%), consolidation (2%), hydropneumothorax (2%), and hyperinflated lung fields with nonspecific shadows (2%). Among the samples collected, 60% of the samples were sputum, and the rest, 40%, were gastric aspirates. CBNAAT was positive in 40 patients (80%). Among sputum samples (n = 30), 76.7% (n = 23) were CBNAAT positive, while among gastric aspirate samples (n = 20), 85% (n = 17) were CBNAAT positive. TST performed gave a positive reading in 78% patients (n=39). Among CBNAAT-positive patients (n = 40), the majority of the patients (85%) were positive for TST. While in CBNAAT-negative patients (n = 10), 50% of patients were positive for TST. Odds of tuberculosis positivity in TST were 17.6% lower compared to Odds of tuberculosis positivity in CBNAAT, and this was found to be statistically significant ($p<0.05$). For tuberculosis diagnosis, sensitivity of the TST test was 85%, specificity was 50%, PPV was 87.2%, and NPV was 45.5%. 70% of CBNAAT-positive patients were unimmunized for the BCG vaccine. While in

CBNAAT-negative patients, 90% of patients were immunised for the BCG vaccine. Odds of positivity in CBNAAT were 48% lower in those who immunised with BCG vaccine, and this was found to be statistically significant ($p<0.05$). Among the sputum or gastric aspirate samples checked for AFB microscopy, 14% (n = 7) were positive. All the samples positive for AFB smear microscopy were sputum, and the AFB smear was negative in all gastric aspirate samples. Among the CBNAAT positive cases, 17.5% of patients were positive for AFB microscopy. Among CBNAAT-positive patients (n = 40) and none-positive for AFB microscopy among CBNAAT-negative patients. For tuberculosis diagnosis: sensitivity (Sn), Specificity (Sp), positive predictive value (PPV), and negative predictive value (NPV) of AFB microscopy compared to CBNAAT were 17.5%, 100%, 100%, and 23.3%, respectively. By applying the chi square test, the relation between CBNAAT and AFB microscopy findings was found to be statistically non-significant ($p > 0.05$) (Table 1).

Table 1. Age wise Positive findings of different test:

Age wise Positive findings of different test									
Type of test	**Age groups (Years)**						**Total**	**Sensitivity Compared to CBNAAT (n=40)positive cases**	**P value applying chi square test**
AGE	**< 1 yr**	**1 – 5yrs**	**6 – 10yrs**	**11 – 15 yrs**	**SPUTUM N=30**	**GA N=20**	**N=50**		
AFB	0	0	1 (14.3)	6 (85.7)	7	0	7 (100)	17.5%	(p>0.05)
CB NAAT	5 (12.5)	18 (45.0)	6 (15.0)	11 (27.5)	23	17	40 (100)	100%	(p<0.05)
PCR	4 (15.4)	11 (42.3)	4 (15.4)	7 (26.9)	17	9	26 (100)	65%	(p<0.05)
Culture	2 (10.0)	9 (45.0)	4 (20.0)	5 (25.0)	13	7	20 (100)	50%	(p<0.05)

Liquid culture was positive in 40% (n = 20) samples. Among sputum samples (n = 30), 43.3% (n = 13) were culture positive, and among gastric aspirates (n = 20), 35% (n = 7) were culture positive (Table 1). Among the CBNAAT positive cases, 20 cases were liquid culture positive, i.e., 50%, but none of the CBNAAT negative samples were culture positive. For TB diagnosis, Sn, Sp, PPV, and NPV of liquid culture compared to CBNAAT were 50%, 100%, 100%, and 33.3%. By applying the chi square test, the difference of results between CBNAAT and liquid culture was found to be statistically significant ($p<0.05$). PCR targeting IS6110 was positive in 52% (n = 26) patients. Among the sputum samples (n = 30), 56.7% (n = 17) were PCR IS6110 positive, and among the gastric aspirates (n = 20), 45% (n = 9) were positive for PCR IS6110. Among the CBNAAT positive cases, 26 cases were PCR IS6110 positive. i.e., 65%, but none of the CBNAAT negative samples were culture positive. For TB diagnosis, Sn, Sp, PPV, and NPV of liquid culture compared to CBNAAT were 65%, 100%, 100%, and 41.7%. By applying the chi square test, the relation between CBNAAT and PCR test findings was found to be statistically significant ($p<0.05$) (Table 2).

Table 2. Comparison of Rifampicin sensitivity with different test

Type of test	**Rifampicin sensitivity**		
	Sensitive	**Resistance**	**Total**
***LPA* (%)**	18 (81.8)	4 (18.1)	22 (100)
***DST* (%)**	17 (85)	3 (15)	20 (100)
CBNAAT	34(85)	6(15)	40(100)

Among CBNAAT-positive patients, 85% (n = 34) patients were rifampicin-sensitive, and 15% (n = 6) patients were rifampicin-resistant. DST was done in all culture-positive samples and showed the SS pattern (i.e., isoniazid-rifampicin sensitive) in 17 samples and the RR pattern (i.e., isoniazid resistance-rifampicin resistance). LPA was done in all culture and smear-positive samples. (n = 22), and the SS pattern (i.e., Isoniazid sensitive-rifampicin sensitive) was obtained in 18 samples; the SR pattern (i.e., Isoniazid sensitive-rifampicin resistance) was found in 1 sample; and the RR pattern (i.e., Isoniazid resistance-rifampicin resistance) was seen in 4 samples. DST and LPA results were in concordance with the resistant pattern obtained in CBNAAT (Table 2).

Discussion

For the past several years, Xpert MTB/RIF, also known as GeneXpert or cartridge-based nucleic acid amplification testing (CBNAAT), has been used more frequently in the diagnosis of pulmonary tuberculosis in highly endemic and poorly resourced countries like India. Limited studies have been conducted regarding the diagnostic use of this newer technique in a highly endemic country like India. Though there are sufficient studies in adult TB cases, there are very limited studies in the paediatric population. Also, comparative studies on resistance detection by means of conventional and molecular methods in samples of the paediatric population are very few. In gastric aspirate and sputum samples that were smear and culture positive, the sensitivity of CBNAAT was 95.6%, which was similar to that reported in fresh clinical samples. As expected, the sensitivity of CBNAAT was superior to that of smear microscopy ($p = 0.0001$). Performance of CBNAAT has been previously evaluated mostly on sputum samples collected from adult TB patients (7–10).

Of the 50 children in the current study, 50% were between the ages of 0-5, 26% were between the ages of 11-15, and 24% were between the ages of 6-10 years. Similarly, Anshu et al. [11] noted 47.6% of patients under the age of five, while Raizada et al. (12) reported 28.9% of patients in the 0–4 age range.

A study by Singh et al. [13] included 403 children under the age of 14, with a median age of 10. Males (56%) dominated the current study with a male-female ratio of 1.2:1. Similarly, male patient predominance was present in the study conducted by Anshu et al. (11) (67% male, M:F ratio 2.1:1) and Raizada et al. (12) (54.7%). However, in the study of 403 children, Singh et al. [13] observed a female (58.2%) predominance.

According to the current study, 80% of patients had a positive contact history, and 98% of patients were malnourished. In the Anshu et al.(11) study, however, only 18.4% of the patients had a contact history. Of the drug-resistant TB patients, Raizada et al. [12] observed that 79.6% had a positive contact history with TB or DR-TB.

The CBNAAT machine is easy to operate, however, and is a little dependent on the user's skills. Though a one- to two-day training program can easily train technicians or routine users to handle CBNAAT. This method yields results within 90 minutes. Concerns about contamination and biosafety are also minimised as compared to AFB microscopy and culture techniques. RIF resistance and M. tuberculosis can be detected simultaneously by CBNAAT in less than two hours, giving it a quick turnaround time. In the current investigation, the Mantoux test turned positive in 78% of the patients, AFB microscopy found MTB in 14% of the patients, liquid culture gave positive results in 40% of the patients, and CBNAAT detected MTB complex in 80% of the patients. Anshu et al. [11], however, reported that 13% of patients had positive mantoux tests, 27.7% were culture positive, 18.9% of patients had MTB detected by AFB microscopy, and 26.4% of the samples only had positive CBNAAT results. In 144 out of 205 lung specimens, Swojanya et al. [14] detected MTB in CBNAAT, while sputum

AFB was able to detect only 108 cases (52.68%), as opposed to 144 (70.24%).

Of the 109 sputum smear-positive patients, CBNAAT confirmed MTB in 108 of them and in 36 of the 96 sputum smear-negative cases. Among 8,370 paediatric presumptive TB and presumptive DR-TB cases between April and November 2014, Raizada et al. [12] discovered that TB detection rates were twofold greater with CBNAAT as opposed to smear microscopy. Out of 30 patients, 58.8% tested positive for Xpert MTB, 63.3% tested positive for MGIT culture, and 29.4% tested positive for AFB microscopy, according to research by Shah and Gupta [16].

In this study, gastric aspirate samples (85%) had more pulmonary TB-positive CBNAAT results than sputum samples (76.7%). Similarly, a study by Anshu et al. [11] found that a sample of gastric aspirate/lavage showed higher positive pulmonary TB by CBNAAT (33%) than a sample of sputum (21.4%). According to Singh et al. [13], 24.4% of the samples tested positive for CBNAAT. 34 samples (85%) of the 40 CBNAAT-positive samples in our study were rifampicin sensitive, while 6 samples (15%) were rifampicin resistant. The CBNAAT results also showed concordance with the LPA and conventional DST results. Anshu et al. [11] reported that out of 105 CBNAAT-positive individuals, 5.7% of them were rifampicin resistant and 94.3% were rifampicin sensitive. Among the 677 MTB-positive CBNAAT samples, Raizada et al. [12] reported 11% of samples were resistant to rifampicin. Rifampicin resistance was detected in 55% of patients among positive Xpert MTB samples, according to research by Shah and Gupta [16].

In the current study, the Sn, Sp, PPV, and NPV values of AFB microscopy were 17.5%, 100%, 100%, and 23.3%, respectively, for tuberculosis diagnosis when compared to CBNAAT. Nonetheless, Anshu et al. [11] reported that the ZN smear's Sn, Sp, PPV, and NPV values in reference to culture were, respectively, 39.09%, 88.85%, 57.33%, and 79.19%. It was clearly evident that Sn, Sp, PPV, and NPV values of AFB microscopy were very poor for diagnosing MTB in a sputum/gastric aspirate sample. CBNAAT would divert treatment burden away from “false cases” to “true” smear-negative TB cases, thereby reducing treatment costs and toxicity in individuals who do not actually have TB while improving treatment accuracy and cost-effectiveness. It also helps identify true TB-negative patients, thus contributing to cost savings by avoiding unnecessary treatment.

52% of the cases in the current study tested positive for the IS6110 gene by PCR, while 48% tested negative. When it came to PCR targeting IS6110, none of the CBNAAT-negative subjects tested positive. In the current study, the sensitivity of PCR targeting the IS6110 gene for tuberculosis diagnosis was 65%, specificity was 100%, PPV was 100%, and NPV was 41.7%. Among the patients sputum sample (n = 30), 56.7% of them had positive PCR results, while 43.3% had negative results. While among the gastric aspirate samples (n = 20), 45% of patients turned PCR positive and 55% PCR negative. Thus, the PCR result yield was higher in the sputum sample than gastric aspirate. There

are no studies yet comparing PCR IS6110 and CBNAAT in paediatric pulmonary samples.

One patient in the current study was positive for HIV. ATT was initiated for the samples that turned positive on CBNAAT. CBNAAT negative cases were considered for alternate diagnosis; one had bronchiectasis, one had bronchial asthma, and three others had latent TB infection. In this study, patchy opacities in the lung parenchyma were observed in 34% of cases and hilar lymphadenopathy in 46% of cases on chest roentography. In a study by Boloursaz et al., hilar lymphadenopathy, with or without lung parenchymal involvement, was the most common finding (15).

Conclusion

Around 17.5% were positive on AFB Ziehl Neelson staining among the n = 40 CBNAAT-positive patients; however, the AFB smear was negative in all CBNAAT-negative cases.

Merely 20 cases, or 50%, had liquid culture positive results out of the CBNAAT negative samples; none showed culture positive results.

Among the 22 samples in which LPA was done and TB bacilli detected, CBNAAT was also positive.

DST and LPA results were in concordance with the resistant pattern identified on CBNAAT.

In 52% of CBNAAT-positive samples, PCR IS6110 was positive; in all CBNAAT-negative cases, it was negative. Clinically, among the patients who were negative on CBNAAT, five responded well to antibiotics, two had an alternate diagnosis, and the remaining three were given INH prophylaxis. On follow-up, none of them developed active tuberculosis.

Accordingly, the results strongly suggest that CBNAAT of sputum/gastric aspirate is 100% specific and very sensitive for diagnosing paediatric pulmonary tuberculosis cases and detecting resistance.

Conflict of interest

The authors declare that they do not have conflict of interest.

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